UNCOVERING NOVEL ACTIONS OF NUMB AND ARYL HYDROCARBON RECEPTOR IN THE PITUITARY

BY

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DISSERTATION

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Abstract

Many physiological responses are regulated by the pituitary including, but not limited to, growth, metabolism, response to stress, and fertility. These responses in the anterior lobe are mediated by the release of the following hormones, growth hormone (GH), adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), prolactin, (PRL), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), and in mice, an additional intermediate lobe produces alpha melanocyte-stimulating hormone (αMSH) from alternative cleavage of the *Pomc* gene. The pituitary modulates many of these processes in a dynamic manner in response to physiological need. This includes not only adjusting hormone secretion, but can also include increasing cellular proliferation to meet greater demands. Taken together with the fact that these hormone producing cells differentiate in a spatial and temporal specific manner, the pituitary then is a convenient model to study cellular differentiation, endocrine cell localization, and progenitor cell maintenance. The present work identifies a novel endogenous function for the gene *Numb*, as well as both intrinsic and extrinsic effects of aryl hydrocarbon receptor activation, on pituitary hormone producing cells.

The adaptor protein Numb was originally described as an important mediator of asymmetric cell division during neurogenesis, however in the study described here it is shown to have an important cell adhesion function in the post-natal pituitary. Both NUMB and related homolog NUMBLIKE, are expressed early in pituitary development before hormone cell specification, at which point they become undetectable by immunohistochemisty. NUMB expression becomes prominent again after birth in the αMSH expressing intermediate lobe cells, as well as the gonadotropes in the anterior lobe. Full *Numb* knockout mice (*Numb*$^{-/-}$) are embryonic lethal before development of the pituitary, so the work described here utilized a Cre-loxP conditional knockout system driven off the *Pomc* promoter to delete Numb and Numblike in the intermediate lobe. Conditional double knockout animals (cDKO) have metaplastic intermediate lobes with impaired localization of adherens junctions proteins including N- and E-cadherin, and beta- and alpha-catenin, and strikingly, infiltration by arginine vasopressin secreting posterior lobe axons. Last, the Sox2 positive progenitor cell niche is disrupted such that these cells become more diffuse throughout the intermediate lobe rather than restricted to

the luminal border of the intermediate lobe, as compared to controls. Numb then is critical for maintaining proper intermediate lobe cell adhesion and localization.

A class of endocrine disruptors includes environmental dioxins, which act by binding the aryl hydrocarbon receptor (AhR). These compounds are known for causing fertility problems and cancer development, yet little is understood regarding the role of AhR in the mammalian pituitary. This work describes the effect of AhR activation on endogenous pituitary hormone expression and proliferation in the GH3 rat somato-lactrotroph cell line. The AhR agonist β-naphthoflavone at nanomolar doses impairs mRNA and protein expression of PRL, but not GH transcription in GH3 cells. Also, when combined with 100 nM of the partial AhR antagonist α-naphthoflavone, the suppressive effects on PRL were enhanced and GH levels remained unaffected. These changes coincide with a suppression of the anti-proliferative signaling cytokine TGFbeta1. In knockout studies, we find that female $AhR^{-/-}$ mice have significantly reduced LH expression at postnatal day 90. Overall, these results show AhR not only has important endogenous effects with respect to normal pituitary function, but also that exogenous stimulation through environmental contaminants could lead to significant suppression of pituitary hormones.

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Chapter One: Introduction

Understanding cell fate determination is important for basic knowledge of normal tissue development, but also has implications for disease such as uncontrolled progenitor cell proliferation and cancer. Coordination of proper lineage commitment involves an immense number of factors each with unique and overlapping functions. However, the protein NUMB, originally identified as critical to organ cell precursor development in *Drosophila* (1), deserves particular attention. Numb is not only a critical factor for cell lineage determination during development, but also contributes to a wide range of other functions that can cause human disease when misregulated. These actions include but are not limited to, cell adhesion, migration, cell cycle control, and adaptor activities for protein degradation and cell membrane tethering.

Numb is an evolutionarily conserved gene with some important phylogenetic differences. Unlike *Drosophila* Numb (d-Numb), which exists in one form, mice (m-Numb) have 4 alternatively spliced isoforms determined by the presence or absence of the PTB domain (PTB_L or PTB_S for long and short) or a proline rich region (PRR) closer to the C-terminus (PRR_L or PRR_S for long and short). These are also sometimes referred to by their molecular weights, which are Nbp72, -71, -66, and -65 where 1 kDa and 6 kDa accounts for the PTB and PRR peptides respectively (Figure 1.1). M-Numb shows 63.3% homology with d-Numb within the first 292 of 593 amino acids including all key residues of a phosphotyrosine binding (PTB) domain, with very little homology in remaining amino acids (2). This suggested a key functional role within the PTB region of Numb. The significance of the PRR domain is less well understood, but PRR-containing forms are enriched in progenitor cells (3-5).

Numb interacts with proteins involved in endocytosis and can be localized to the plasma membrane and intracellular vesicles. The PTB insert (PTBi) is required for Numb localization to the plasma membrane (6, 7), and in the absence of PTBi, Numb expression is diffuse throughout the cytoplasm(8). This is supported with data showing isolated PTBi segments localize to the plasma membrane, yet are not found on vesicles compared to the full length Numb protein (9). Protein binding regions within Numb implicated in endocytosis include two binding domains for alpha-adaptin and an Eps15 homology (EH) containing sequence in the C-terminal region (10, 11). The Eps homology domain

includes NPF binding motifs (asparagine-proline-phenylalanine), which are important for interactions with critical endocytic protein complexes including proteins within the Epsin15 family, involved in cathrin-dependent as well as clathrin-independent endocytosis (10-13). Site-directed mutagenesis analysis shows that mutating either the first C-terminal alpha-adaptin binding site (DPF) or EH domain disrupts binding to Eps15, but does not affect Numb membrane or vesicle localization (9, 12). However, removing the C-terminal 38 amino acids containing both DPF sites and EH domain, reduced vesicle-associated Numb and caused a generalized cell membrane localization (9). This suggests the C-terminal alpha-adaptin and EH domains may work cooperatively and are required for vesicle binding. Overall, Numb is an adaptor protein with functions requiring multiple protein interactions including, but not limited to, facilitating membrane localization and proteosome trafficking of membrane proteins.

There is also a mammalian homolog to Numb, Numblike, that has 76% identity to m-Numb between amino acids 42 and 331. Expression of Numblike in mice is predominantly limited to the embryonic and adult nervous system, specifically in post-mitotic cortical neurons. This contrasts with Numb, which is exclusively found in neural progenitors within the ventricular zone during neurogenesis (14), indicating there could be some overlap in function, but also that there could be distinct roles for each gene.

Numb function *in vivo*, *Drosophila*:

A better understanding of the protein actions of Numb in mammalian systems should first begin with an introduction to its discovery and characterization in *Drosophila*. During neurogenesis in mammals and invertebrates, a very rapid expansion and differentiation of daughter cells is tightly controlled and necessary for proper nervous system development. While asymmetric division is a cardinal and critical process for the development of multiple cell lineages from a single progenitor precursor, symmetric divisions are also important to maintain an active progenitor pool and sufficient numbers of future differentiated cells. Studies in *Drosophila* show that differential or asymmetric segregation of Numb during division of sensory organ precursor (SOP) cells is required for the proper establishment of all subsequent differentiated cell types (1, 15, 16). During SOP division a single precursor will yield 4 daughter cells following two divisions. The

first division involves an asymmetric division to produce one cell that will divide further symmetrically and ultimately produce two outer support cells, while the other precursor daughter cell will asymmetrically divide to yield a sheath cell and neuron. During precursor division, NUMB preferentially localizes to one half of the membrane and is inherited by the cell ultimately responsible for producing the neuron and sheath cell. In fact, loss of *Drosophila Numb* (d-Numb) results in extra support cells concomitant with loss of sheath and peripheral neurons (1, 16). Interestingly, introduction of not only d-Numb but also m-Numb can rescue the sheath cells and peripheral neurons of sensory organs. Further, m-Numb co-localizes asymmetrically with d-Numb in SOP cells. Cells inheriting NUMB following division may therefore be adopting alternate cell fates due to the actions of NUMB in receptor internalization and interactions with endocytic machinery. These early *Drosophila* studies were important to identify Numb and show its role in neural cell fate choice through asymmetric distribution.

Numb function *in vivo*, mouse:

So far the greatest interest has been focused on Numb actions during neurogenesis, however it must be mentioned that evidence is rapidly emerging that Numb also plays a critical role in other organ systems as well. The first mouse knockout model of *Numb* showed embryonic lethality at e11.5. These mice have neural tube defects as well as precocious forebrain neuron production (17). Interestingly, this phenotype came from a targeted deletion including only exons 5 and 6, critical coding sequences for the PTB domain, and similar results came shortly after by another group having generated a full *Numb* knockout by deleting the ATG start site (18). These mice also died around e11.5 with neural tube defects, however they were also reported to have angiogenic defects and possible placental dysfunction. In contrast to the PTB deletion, the full *Numb* knockout mice did not show evidence of enhanced forebrain or midbrain neurogenesis and in fact had reduced hindbrain development in addition to impaired peripheral sensory neurons. These results suggest the PTB region is critical for early embryogenesis, particularly neural tube formation, while other regions of *Numb* outside of the PTB sequence may be partly responsible for the reduced hindbrain formation and angiogenesis defects.

As previously mentioned, Numblike is distinct from Numb in that it is expressed and inherited symmetrically and appears to be limited in expression to post mitotic neurons in contrast to progenitor cells (14). The *Numblike* knockout therefore might be predicted to have a very different phenotype than the *Numb* knockout, however this is not the case. The *Numblike* knockout mouse has no obvious phenotype with the exception of lower fertility in females, however the double *Numb, Numblike* double knockout mouse shows similar but more severe phenotype compared to the *Numb* single mutant mouse and so the two genes are treated as functionally redundant (19).

Following the discovery of embryonic lethality of the *Numb* knockouts and neural tube defects, three separate neural conditional *Numb* knockout mice were analyzed, yielding conflicting results. The first published knockout utilized the early (approximately e8.5) progenitor expressing nestin-Cre recombinase to mediate *Numb* and *Numblike* excision (19). These mice have an insufficient maintenance of neuronal precursors, shown by reduced numbers of BrdU-labeled cells and decreased expression of the progenitor marker Hes5. Further, there is a substantial increase in expression of the neuronal differentiation marker Hu in these conditional double (*Numb Numblike*) knockout (cDKO) mice (19). Paradoxically, cDKO mice using an Emx-Cre mediated excision (expressed e9.5 to e12.5) show progenitor hyperproliferation with impaired differentiation and delayed cell cycle exit (20). Last, conditional deletion of *Numb* on a *Numblike* null background using the D6-Cre beginning at e10.5 in dorsal forebrain progenitor cells again shows premature progenitor cell depletion (21). Together, these studies support a role for *Numb* in progenitor maintenance but also for promoting cellular differentiation. This is sustantiated by experiments showing that Numb can be segregated to neuronal progenitor cells following asymmetric division (2) or to differentiated neurons (22). Explanations to account for these disparities could include that the timing of *Numb* deletion is an important consideration, particularly with respect to isoform expression. Data show that during neurogenesis PRR_L is predominant between e7 and e10 and diminishes by e13 while PRR_S is detectable throughout neurogenesis and into adulthood (3, 8, 23). The PTB region shows a similar trend such that the expression of the shorter form is more dominant in mature neurons compared to progenitors (3) and so the phenotypes observed may be reflective of deleting differing isoform expressions at

different times and tissues. Also, conditional deletion within different cell types may affect cellular organization, and mislocalized cells may indirectly affect maintenance of the progenitor cell niche (20, 21). Overall, NUMB can promote as well as inhibit progenitor cell maintenance, and these functions may be due in part to its function as an adaptor protein which facilitates multiple protein interactions.

Numb induces Notch degradation

While far from well understood, significant progress has been made in recent years regarding Numb isoform and conserved protein binding domain functionality. The characterization of the PTB region in d-Numb provided insight into how the mouse isoforms containing or lacking the PTB sequence affects cellular function. The peptide structure suggests it can bind a broad array of distinct peptide ligands (24, 25) however some of the first evidence to suggest a specific function came from in vitro studies showing that m-Numb can bind regions of the Notch intracellular domain (2, 26). Notch activity directs cell fate determination, progenitor cell maintenance, proliferation, differentiation and apoptosis (27). An important developmental pathway, Notch signaling occurs when neighboring cells expressing transmembrane ligands in the Delta and Serrate/Jagged family bind the transmembrane Notch receptor, induce the intracellular gamma-secretase cleavage of the intracellular domain (NICD), which then translocates to the nucleus and with other factors initiates transcription of downstream transcription factor harry and enhancer of split (Hes and Hey) related genes which generally inhibit differentiation (28, 29). It might be expected then, that loss or gain of NICD function through alterations in Numb could in turn skew the final distribution of differentiated cells.

Numb associates with endocytic machinery and can bind the Notch ICD though the PTBi. There is now evidence that Numb can directly initiate the degradation of the Notch receptor through interactions of the PTBi with the ubiquitin ligase Itch, which causes subsequent polyubiquitination and degradation of Notch ICD (13, 30). Specifically, protein mutants lacking the critical Itch binding site in the PTB region (NbΔPTBC) or the C-terminal 41 amino acids including the DPF and NPF regions (NbΔC) had no effect on Notch levels at the membrane compared with wild type p66

Numb. Interestingly, in contrast to wild type Nbp66 which dramatically reduces intracellular levels of Notch, NbΔPTBC caused increased levels of intracellular Notch. An NbΔC form lacking DPF and NPF sites but intact PTBi, had very little effect on overall Notch intracellular levels. In fact, Itch has been shown to facilitate Notch trafficking to lysosomes (30-32). As expected then, internalization and recycling of Notch to the membrane is constitutive, and both overexpression and RNA interference of Numb did not alter Notch internalization. This suggests that Numb is not principally involved in Notch receptor internalization, but mainly traffics NICD out of the constitutive recycling process towards endosomes, primarily though interactions with Itch.

Due to the evidence ascribing Notch activation with proliferation and inhibition of differentiation, it was assumed for a long time that the Notch inhibitory actions of Numb were responsible for both cell fate determination and progenitor cell maintenance through interactions with the PTB region. However, given the evidence that Nbp66 Numb, which does not contain the PRR sequence, causes Notch ICD ubiquitination and trafficking to endosomes, the PRR region may play a more critical role in progenitor renewal. An important structure-function study examined the effects of anti-sense morpholino *Numb* knockdown, with re-expression of separate isoforms (Nbp72, 71, 66, 65) in embryonic cortical progenitors at e13.5 and from P19 neurons (3). Interestingly, only expression of the PRR_L isoforms (Nbp72, 71) caused an increased number of neuronal progenitors marked by Nestin compared to EGPF transfected controls. Similarly, expression of either PRR_S isoforms (Nbp65, 66) reduced Nestin positive cells and increased the number differentiated cells marked by increased differentiation marker TUJ1. Of particular importance, these actions of progenitor renewal and differentiation are Notch-independent, such that there was no difference between isoforms in downstream *Hes1* or NICD cofactor CSL activity by luciferase assay. However Numb can alter Notch in these conditions as *Numb* knockdown showed strong luciferase activation of both *Hes1* and CSL. Last, re-expression of PRR_S isoforms not only induced activation of differentiation marker *Mash1*, but also substantially increased neurite outgrowth, in contrast to PRR_L forms. This “isoform switching” as its become known has been described during other tissue development as well, particularly endocrine organs. During pancreas development

before the divergence of differentiated endocrine and exocrine cells, both PRR_S and PRR_L isoforms are co-expressed. However, as endocrine glands begin to differentiate the PRR_L forms diminish while expression of the PRR_S isoforms is sustained (5). Also in adult mouse testes, Nbp71 is expressed exclusively in germ cells, while somatic cells only express PRR_S isoforms (4). This all suggests that the cell lineage determination role of Numb may be mediated by trafficking Notch ICD for degradation, while the PRR plays a still unknown critical role in progenitor cell maintenance, which may be independent of Notch signaling events.

Numb controls cell adhesion

Clearly, Numb has many functions that extend beyond Notch involvement, particularly at the cellular membrane. Of particular interest in recent years is the involvement of Numb in interacting with cadherins, integrins and actin cytoskeletal components. The PTB and C-terminal regions of Numb have been described to bind E- and N-Cadherin as well as alpha- and beta-catenin, and are required for maintaining proper cellular localization of these proteins and of adherens junctions (33). Also, NUMB is critical for adhesion of radial glial cells and proper localization of neuronal progenitors (33). Cell adhesion is a critical component of migration, and in a paradoxical role of maintaining adherens junctions, NUMB also binds integrin-β proteins and facilitates endocytosis of these proteins with clathrin coated pits. Downregulation of Numb by RNAi reduces integrin endocytosis and impairs migration (34). This process is normally regulated with phosphorylation of Numb by aPKC, thus polarization by Par and aPKC proteins may not only affect cell fate determination through interruptions in Notch signaling, but also promote integrin endocytosis and leading edge migration (34).

Numb and cell cycle control

Proper control of proliferation and cellular differentiation are critical early events in organogenesis. Thus, tumor growth and the development of cancers are particularly sensitive to aberrations in proteins such as Numb during development or progenitor maintenance in adulthood. Numb has been linked to cell cycle control in several contexts, one of which includes binding to the Golgi apparatus protein ACBD3 during mitosis.

Through an N-terminal sequence, Numb binds ACBD3 and facilitates dispersion of Golgi cisternae during interphase, a critical aspect of cell cycle progression (35). Numb can also affect the cell cycle in a more direct manner by altering the stability of tumor suppressor p53. In contrast to the role Numb plays in promoting ubiqtuitin-mediated degradation of Notch ICD, it has the contradictory role of preventing ubiquitination of P53. This occurs through direct inhibition of the ubiquitin ligase MDM2 (36). Numb not only enters a trimeric complex with p53 and MDM2 to stabilize and enhance p53 activity, but loss of NUMB in human breast tumors is associated with decreased levels of p53, enhanced chemoresistance, and poorer disease prognosis (36). Loss of NUMB has been described in 50% of human mammary carcinomas within a group of breast cancer patients, clearly highlighting its role in maintaining cell cycle progression (37).

In addition to breast cancer, loss of NUMB has been associated with brain and lung cancers. During normal brain development, the Hedgehog signaling pathway promotes neuronal progenitor renewal, and aberrant Hedgehog activity, via enhanced downstream target *Gli1*, results in medulloblastoma (38, 39). More recently, it has been shown that enhanced GLI1 activity in these medulloblastoma cells is due to loss of NUMB in cerebellar granule progenitors, which disrupts normal Itch mediated degradation of GLI1 (40). Further, loss of Numb is associated with small cell lung carcinomas, where approximately 30% show increases in Notch-mediated signaling events (41). This demonstrates Numb can alter proliferation in many contexts, and its loss is associated with aggressive cancers, only some of which involve altered Notch signaling.

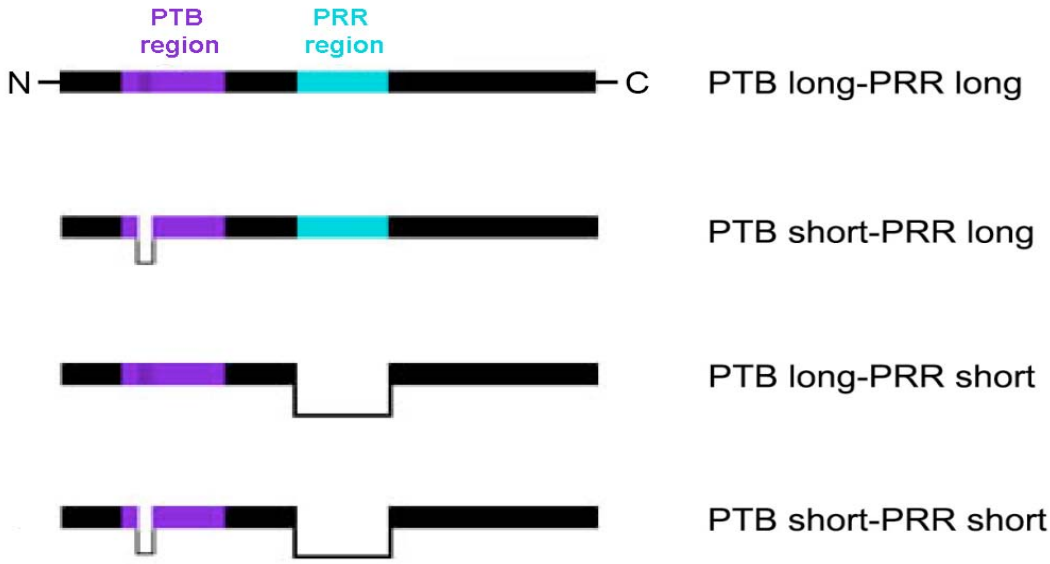
An understanding of Numb in mammalian physiology has come a long way since its first reported role in Notch inhibition and cell fate determination though asymmetric division. Importantly, the vast diversity of protein interactions identified are not exclusive to neurogenesis, but occur during the development of many other tissues, and for convenience, a summary of the understood protein binding domains are illustrated in Figure 1.2. The past two years represent the vast majority of the discoveries of the diverse cellular functions of Numb, including its importance in human disease. Undoubtedly, many new functions await discovery. Chapters 2 and 3 in this present work entitled *Numb, an endocytic adaptor protein, is critical to maintain cell adhesion in the*

*intermediate lobe of the mouse pituitary***,** and *Numb, an endocytic adaptor protein, is highly expressed in pituitary gonadotropes and may influence fertility*, respectively, will attempt to identify a previously unknown role for Numb during mouse pituitary development as well as maintenance of adult hormone secreting cells. With the knowledge that NOTCH2 is transiently expressed during early pituitary development before hormone cell specification (42) and transgenic overexpression of *Notch2* delays gonadotrope differentiation (43) we hypothesize that Numb may be required to control and inhibit Notch activity following early pituitary organogenesis. Second, cadherin expression in the adult pituitary is a critical component of hormone secreting networks (44, 45), so we expect NUMB might be required for proper adherens junction formation, and proper migration of fully differentiated cells. Third, Numb was identified in a cDNA microarray from adult pituitary progenitor/stem cells sorted based on expression of Sca1. Adult Sca1 low cells express markers of early progenitors, including Sox2 and Sox9, as well as Hes downstream Notch target genes. Numb is enriched in the Sca1 high cell fraction (46). We think Numb may normally restrict proliferation of these adult progenitor cells by inhibiting Notch signaling. Last, given the importance of PRR isoform expression during early organ development and presence of PTB region in many contexts, we expect to find an "isoform switch" such that Nbp72 and 71 predominate early before hormone specification, and are replaced with Nbp 66 and 65, with higher concentrations of PTBi isoforms (Nbp66) near side populations expressing Notch target genes.

The fourth chapter is this work entitled *Aryl hydrocarbon receptor activation affects pituitary hormone cell function* in vivo *and* in vitro, addresses a separate, but potentially linked pathway, that may exert unique actions in pituitary development and function. Interestingly, despite its well-defined role in modulating dioxin responses, the aryl hydrocarbon receptor (AhR) has been reported to have important interactions with Notch in other tissue contexts. The potent AhR ligand TCDD can induce expression of Notch downstream target HES1, at the mRNA and protein level in human mammary carcinoma cells, and AhR activity drives proper IL22 secretion in CD4+ T cells, in a Notch dependent manner (47, 48). While Notch does have important actions during pituitary development as described previously(42, 43), it is intriguing to speculate AhR

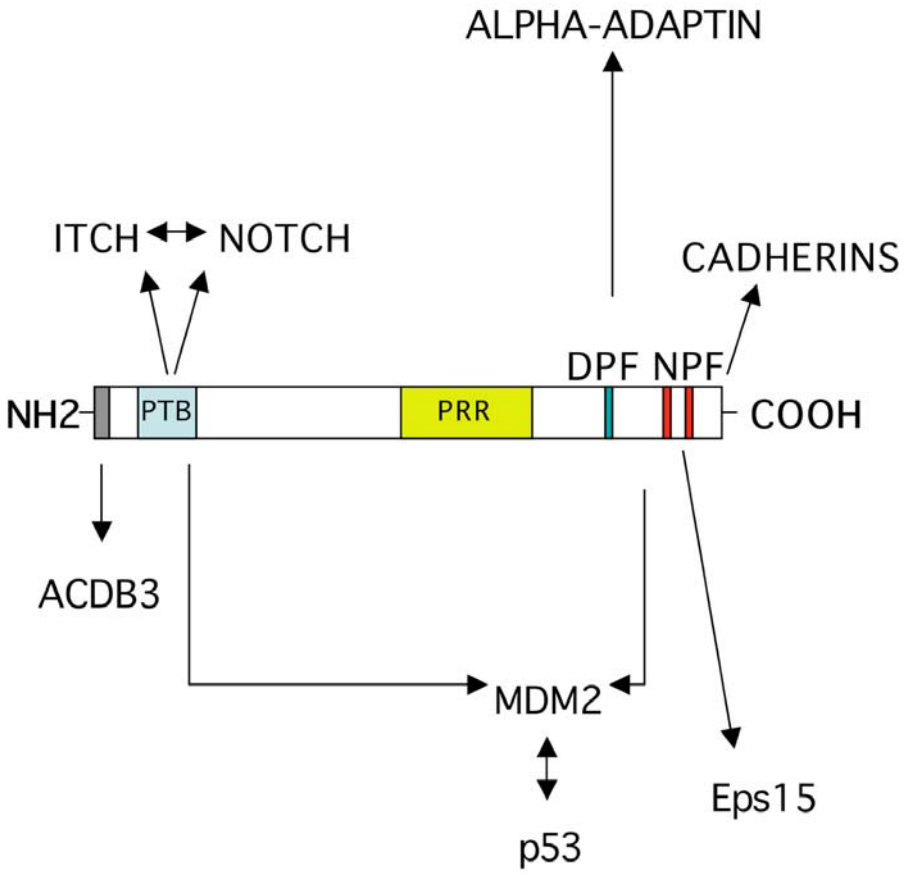
may directly alter pituitary cell function. Given that AhR and the important aryl hydrocarbon interacting protein (AIP) are both expressed in human pituitaries at the protein level, and mutations in *AIP* are associated with pituitary adenomas (49-51), we speculate AhR may play an important endogenous role in pituitary hormone cell development and responsiveness. Further, there have been a few reports describing altered pituitary hormone levels following AhR agonist administration in various animal and tissue culture models, including changes in GH, PRL, ACTH, LH and FSH (52-55). However, some of these results are conflicting and warrant further study. The work described here will attempt to clarify the endocrine disrupting effects of AhR activation on pituitary hormone synthesis utilizing a cell culture method, as well as investigate any changes in postnatal pituitary hormone cell function by analyzing $AhR^{-/-}$ pituitary hormone mRNA levels. We hypothesize levels of the AhR agonist β-naphthoflavone sufficient to induce downstream target *Cyp1a1*, will alter GH and PRL mRNA as well as protein levels in the GH3 rat somatolactrotroph cell line. Also, *in vivo* analysis showing mRNA changes in $AhR^{-/-}$ pituitaries should compliment the cell culture data. Last, given that AIP is believed to restrict AhR transcriptional activity, and that AIP mutations are associated with human pituitary adenomas, we would expect to find increased proliferation following β-naphthoflavone treatment of pituitary cells in culture.

Figure 1.1



Major *Numb* alternative isoforms

Figure 1.2



Structure and identified functional domains of NUMB. PTB = phosphotyrosine binding domain; PRR = proline rich region.

Chapter Two: Numb deletion in POMC expressing cells impairs pituitary intermediate lobe cell adhesion, progenitor cell localization, and neuro-intermediate lobe boundary formation.[1]

Abstract

The pituitary gland contains six distinct hormone secreting cell types that are essential for basic physiological processes including fertility and responding to stress. Formation of hormone secreting cells during development relies on Notch signaling to prevent progenitors from prematurely differentiating. The nature of the signal curtailing Notch signaling in the pituitary is unknown, but a good candidate is the endocytic adaptor protein NUMB. NUMB targets Notch for proteolytic degradation, but it also has a broad range of actions, including stabilizing adherens junctions through interactions with cadherins and influencing cell proliferation by stabilizing expression of the tumor suppressor protein p53. Here, we show NUMB and its closely related homolog NUMBLIKE, are expressed in undifferentiated cells during development and later in gonadotropes in the anterior lobe and melanotropes of the intermediate lobe. All four isoforms of NUMB, are detectable in the pituitary, with the shorter forms becoming more prominent after adolescence. Conditionally deleting *Numb* and *Numblike* in the intermediate lobe melanotropes with *Pomc* Cre mice dramatically alters the morphology of cells in the intermediate lobe, coincident with impaired localization of adherens junctions proteins including E-cadherin, N-cadherin, Beta-catenin and Alpha-catenin. Strikingly, the border between posterior and intermediate lobes is also disrupted. These mice also have disorganized progenitor cells, marked by Sox2, yet proliferation is unaffected. Unexpectedly, Notch activity appears normal in conditional knockout mice. Thus, Numb is critical for maintaining cell-cell interactions in the pituitary intermediate lobe that are essential for proper cell placement.

[1] This chapter to be published in *Molecular Endocrinology* (Moran TB, Goldberg LB, Serviss SL, Raetzman LT (2011), copyright 2011, The Endocrine Society. Permission to reprint this article as a Ph.D. dissertation chapter was granted to Tyler Moran by The Endocrine Society.

Introduction

The pituitary is considered the master endocrine gland and is a convenient system to study cell fate determination due to the temporal and spatial separation of hormone producing cell differentiation during development. In the adult, pituitary hormone cell number can be altered in response to physiological need, suggesting a tight control of progenitor/stem cell maintenance and mobilization is necessary to be maintained throughout the life of the animal (56). The five hormone secreting cell types in the anterior pituitary, with the hormone they secrete, include corticotropes (adrenocorticotrophic hormone, ACTH), thyrotropes (thyroid stimulating hormone, TSH), somatotropes (growth hormone, GH), lactotropes (prolactin, PRL), and gonadotropes (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]). In mice, an additional intermediate lobe exists, and the hormone-secreting cell type is the melanotrope, which produces alpha melanocyte-stimulating hormone (αMSH) (57, 58). ACTH and αMSH are alternate cleavage products of the *Pomc* gene. All cell types in the anterior and intermediate lobe develop from a common precursor primordium within the oral ectoderm called Rathke's Pouch (RP), while the posterior lobe is derived from neural tissue and contains glial cells known as pituicytes and axonal projections from the hypothalamus, which secrete oxytocin and arginine vasopressin.

In mice, RP begins to invaginate and early differentiation markers are detectable at embryonic day 9.5 days (e9.5), including LIM homeodomain transcription factors Lhx3, Lhx4 and Isl1, among others (59-61). Subsequent development of the pituitary involves an orchestration of many major signaling pathways important in other tissues during embryogenesis, including fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), Wnt proteins, Hedgehog factors and Notch receptors and ligands (42, 43, 62-68). These pathways are all critical for induction of the pituitary from the oral ectoderm as well as proper gland structure and size, however coordination of lineage determination remains largely unknown. Presently, one of the best-characterized cell lineage determinants includes the transcription factor Prophet of Pit1 (Prop1). Mutations in *Prop1* cause a postnatal dwarf phenotype (69-71) and a reduction in the Pit1 lineage hormone producing cells: thyrotropes, somatotropes, and lactotropes. Interestingly, NOTCH2 expression is dramatically reduced in the pituitary of *Prop1* mouse mutants

(42) . Notch signaling also appears to directly regulate *Prop1* expression. Loss of the essential Notch cofactor *Rbp-J* (CBF1) impairs Pit1 lineage development, due to loss of *Prop1* and results in a premature appearance of the corticotrope lineage (67). A similar acceleration of corticotrope development is observed only when both *Prop1* and the prototypical Notch target gene *Hes1* are lost (72). Interestingly, complete *Hes1* knockouts have a replacement of melanotropes in the intermediate lobe with somatotropes, suggesting proper intermediate lobe development is also Notch-dependent (68). Finally, a reduction in Notch signaling is necessary for final differentiation of post-mitotic PIT1 cells and gonadotropes (67) (43). Notch signaling therefore cooperates with *Prop1* and modifies its expression level. This ultimately influences pituitary cell differentiation in the PIT1 lineage, gonadotrope development timing, and corticotrope and melanotrope identity.

Notch signaling pathway components expressed in the developing pituitary include the receptors Notch 2 and 3, the delta-like 1 ligand and downstream targets *Hes* and *Hey* genes, and are detectable from early pituitary formation in the mouse at e9.5 but diminish by e13.5-14.5 (42, 67). Recent data suggests that Notch signaling becomes active again in the adult pituitary and may be present in an adult pituitary stem cell side population (73, 74). This population of multipotent cells are Sox2 positive and can differentiate into several hormone producing cells in culture, which represent a putative source by which hormone producing cells can be generated in the adult during times of physiological stress (46, 75, 76). Tight regulation of Notch activity, then, is important for cell fate determination in progenitor cells, including, potentially, stem cells, and improper Notch activation has been implicated in tumor formation (77, 78). The protein Numb is a Notch antagonist and represents a strong candidate for controlling Notch activity in the pituitary.

The adaptor protein Numb was first identified as an important mediator of asymmetric cell division in *Drosophila*. Through interactions with the partition defective (Par) complex including Par3, Par6 and atypical protein kinase C (aPKC), *d*-NUMB is preferentially segregated to, and necessary for, the differentiation of one of two sensory organ precursor daughter cells (16, 79). There is also evidence to suggest asymmetric Numb segregation following mitosis in mammals. During neurogenesis in mice,

asymmetric divisions producing a neuron and progenitor cell showed asymmetric localization of Numb to the differentiating cell. However, during symmetric divisions producing two neuron daughter cells, Numb was segregated to both daughter cells (22). One hypothesis is that cell fate is altered in progeny inheriting Numb. Numb induces degradation of the Notch receptor, thereby inhibiting Notch signaling, and promoting cell differentiation. When Numb was conditionally deleted in developing neurons, however, roles for promoting as well as inhibiting progenitor cell maintenance were uncovered (19-21). It has been suggested these different roles of Numb may also be due in part to the expression of multiple isoforms, two of which differ in sequence in the phosphotyrosine-binding (PTB) domain, and two which have alternate sequences in a proline-rich region (PRR). The functions of these regions are only beginning to be understood but evidence suggests the longer PTB form is important in degradation of the Notch intracellular domain though interactions with ubiquitin ligase Itch (13, 30), as well as localizing NUMB to the plasma membrane (8) and interactions with integrin and cadherin proteins (34, 80). Additional progenitor maintenance properties of Numb may be directed by the longer PRR isoforms. During early mouse pancreas development before endocrine cell specification, both long and short forms of PRR are present, however, following the onset of endocrine cell specification, expression of the long PRR isoform is drastically reduced (5). Similarly, in mouse male germ cells, the longer PRR form is highly expressed compared to a germ cell depleted testis (4). Lastly, in mouse cortical neuron cultures, over-expression of Numb lacking the PRR region correlated with higher levels of differentiation transcription factor MASH1, and also enhanced neurite outgrowth compared with forms containing PRR (3). Together, this suggests the PTB region is important for cell adhesion and membrane localization while the PRR region, by some yet undetermined mechanism, influences progenitor maintenance and differentiation.

In part due to recent developments of identifying protein binding domains and isoform variants, it has become clearer that Numb has a broad range of functions that extends beyond restraining Notch signaling during asymmetric division, which may help to explain its diverse roles in many developmental and adult contexts. Numb interacts with other proteins in addition to Notch to regulate their ubiquitination status. Importantly, loss of NUMB is associated with cancers of salivary gland, lung, and breast,

many of which are due to increased p53 degradation (36, 37, 81, 82). NUMB also interacts with cadherins and is necessary for proper cadherin membrane localization and cell adhesion (33), and evidence suggests NUMB partially controls epithelial to mesenchymal like transitions and alters cell migration as well (80). Therefore delineating the functions of NUMB is important not only for understanding progenitor cell maintenance during development, but also adhesion and cell cycle control in adulthood.

The present study examines Numb protein expression during mouse pituitary development and in adulthood. We show that NUMB and NUMBLIKE are expressed early in pituitary development before hormone cell specification, and that all isoforms are detectable before birth. These isoforms also exhibit dynamic expression patterns such that the shorter PTB and PRR forms are more prevalent than longer forms into adulthood. Interestingly, conditionally deleting *Numb* and *Numblike* from the developing corticotropes and melanotropes, has identified a seemingly Notch-independent function for these proteins in the intermediate lobe. Specifically, loss of *Numb* and *Numblike* shows the importance of these genes in maintaining cell adhesion proteins, facilitating proper localization of Sox2 positive progenitor cells, and establishing the posterior-intermediate lobe boundary.

Results

Numb Expression Patterns in the Developing Pituitary

In order to visualize the expression of NUMB during the course of pituitary development and localize its expression, whole embryos were sectioned and stained using immunohistochemistry. NUMB and NUMBLIKE immunoreactivity were examined beginning from e9.5, where strong staining could be detected throughout Rathke's Pouch (RP) (Figure 2.1A,D). Positive staining is readily observed in the diencephalon as well, particularly in the ventricular zone (arrow, 1A). The veracity of NUMB immunoreactivity was confirmed by a second NUMB antibody as well as *in-situ* hybridization. NUMB protein (Figure 2.7A) and mRNA (Figure 2.7C) appear sporadically expressed throughout RP in midsagittal sections at e10.5, whereas in more peripheral sections, NUMB protein (Figure 2.7B) and mRNA (Figure 2.7D) are more limited to the outer border of the gland. At e11.5, NUMB and NUMBLIKE expression

appear reduced in RP and only a few positive cells are observed in the diencephalon (Figure 2.1B,E). NUMBLIKE expression is still observed at the outer border and luminal side of RP, and less so in the area to become the future anterior lobe (AL). One day later, at e12.5, NUMB staining is similar with the exception of some positive cells at the border between the future intermediate lobe and posterior lobe (PL, Figure 2.1C). NUMBLIKE staining is also similar at e12.5 with positive staining at luminal and outer borders and very little around the developing anterior lobe (Figure 2.1F).

Numb isoform switch during development

There are at least four isoforms of NUMB, each displaying a dynamic expression pattern in tissues during development and in tumor formation. These isoforms are distinguished by the presence or absence of a 48 amino acid sequence in the proline rich region (PRR) and the presence or absence of an 11 amino acid sequence in the phosphotyrosine binding (PTB) region (Figure 2.2). Due to the fact the antibodies used in this study recognize sequences outside of the PTB and PRR, to investigate isoform expression during pituitary development, we performed reverse transcriptase PCR on cDNA prepared from samples from embryonic day 16.5, postnatal day 1 and 9 and finally the adult pituitary.

We observed that during development there is a switch in isoform expression. For the PRR isoforms, the PRR_S is present in the pituitary at all ages examined (Figure 2.2). The PRR_L isoform is most prevalent at e16.5 and P1. We begin to see a decline in PRR-long at P9 and it is detectable only at low levels in the adult pituitary. For the PTB isoforms, at embryonic day 16.5 and P1, the PTB_S and PTB_L isoforms appear expressed at approximately equal levels (Figure 2.2). At P9, the expression pattern shifts so that the upper band, representing PTB_L is diminished. In the adult pituitary, there is still the diminished expression of the upper band, however, there also appears to be increased expression of the lower band, representing PTB_S. Data from a recent microarray analysis of adult pituitaries identified Numb in a sub-population of cells and partially supports our findings that Numb is expressed in the adult pituitary(46).

Numb and Hormone Co-Localization

Numb has been shown to play a role not only in embryonic development, but also in adult tissues, therefore, we examined the expression of NUMB in the adult pituitary. In the mature adult anterior pituitary there are five cell types, each of which secrete a specific hormone: ACTH, GH, TSH, LH, PRL, or FSH. NUMB (red) co-localizes with many, but not all, LHb and FSHb (green) producing cells (Figure 2.3A,B, open arrowhead). There are some NUMB negative and LHb and FSHb singly positive cells (Figure 2.3A,B, arrow), as well as some NUMB positive and LHb and FSHb negative cells (Figure 2.3A,B, closed arrowhead). NUMB is not detected in any other hormone-producing cell in the anterior lobe (Figure 2.3C-F). Co-stains with alpha glycoprotein subunit (α-GSU, dimerization partner for TSHb, FSHb and LHb) show some co-localization with NUMB, and provide further evidence NUMB is expressed in gonadotropes (data not shown). NUMBLIKE was not detected in hormone producing cells of the anterior lobe by immunostaining (data not shown).

***Pomc* Cre specific deletion of *Numb* and *Numblike* alters adhesion and boundary formation**

From the hormone colocalization data we were able to identify that NUMB (red) is present in many POMC expressing cells (green) of the intermediate lobe (Figure 2.4A), despite a lack of NUMB staining in POMC cells of the anterior lobe. We did not observe any NUMBLIKE immunostaining in the intermediate lobe. Using this information we employed a *Pomc* Cre to selectively knock out *Numb* and *Numblike* in these POMC expressing melanotropes in the pituitary (18, 83). In addition to the intermediate lobe, *Numb* and *Numblike* will also be eliminated from the *Pomc* containing neurons in the hypothalamus. However, POMC neurons are not known to innervate or directly affect intermediate lobe cells, which are instead innervated by dopaminergic, serotonergic, and GABA-ergic neurons (84-88). Further, POMC neurons of the arcuate nucleus are of different developmental origin, and can function independently from the pituitary. Mice deficient in *Mash1* show a lack of POMC neuron differentiation despite normal POMC expression in the pituitary (89). Also, loss of glucose sensing in POMC neurons results in impaired glucose tolerance, yet appears to have little consequence on pituitary function as

shown by normal corticosterone levels (90). These data suggest any pituitary effects observed in these *Numb* and *Numblike Pomc* Cre conditional knockout, should be intrinsic.

We examined the adult pituitary of conditional double knockout (cDKO) (*Numb fl/fl Numblike fl/fl Pomc* Cre) or control (*Numb fl/fl Numblike fl/fl)* littermate mice for hormone staining as well as morphological differences. In cDKO intermediate lobes at postnatal day 30, we find that the melanotropes are specified in the absence of *Numb* (Figure 2.4B, green) and that NUMB expression is absent from almost all melanotropes (Figure 2.4B, red). On occasion, a large NUMB/POMC double-labeled cell can be observed in the cDKO intermediate lobes (box, 2.4B). The cDKO melanotropes also appear disorganized as confirmed by H&E stain (Figure 2.4C,D) and are less dense. Manual cell counts of DAPI stained nuclei reinforced the observation that the cDKO intermediate lobes had significantly fewer cells per area (42± 2.8 and 34 ± 2.1 cells per square inch for control and cDKO respectively; $P < 0.05$). As expected, NUMB expression is maintained in the anterior lobe of cDKO animals (Figure 2.4B), as NUMB does not colocalize with ACTH producing cells. The conditional knockout strategy includes one loxP site upstream of the ATG initiation codon and one loxP site within part of the PTB sequence (Figure 2.4E). The antibodies used in this study recognize NUMB peptides downstream of the PRR. Also shown is confirmation of deletion at the genomic level by PCR from DNA isolated from whole pituitaries (Figure 2.4E).

Knowing that NUMB is important for maintaining adherens junctions in radial glial cells(33), several adhesion markers were investigated by immunohistochemistry in the cDKO pituitaries. Compared to control samples (Figure 2.5A), cDKO intermediate lobes have dysregulated E-CADHERIN expression (Figure 2.5B). Notably, there appears to be increased E-CADHERIN expression throughout the intermediate lobe, yet it is more diffuse and less localized to the membrane than the restricted border of E-CADHERIN positive cells in the control. In contrast to upregulated E-CADHERIN, other components of adherens junctions appear dramatically downregulated. In the control, N-CADHERIN is present on the membrane of virtually all cells in the intermediate lobe, as well as the PL and AL (Figure 2.5C). In contrast, the cDKO has a loss of membrane N-CADHERIN expression exclusively in the intermediate lobe cells (Figure 2.5D). A similar pattern is

seen for BETA-CATENIN, localized to the cell membrane of control IL cells (Figure 2.5E), compared with a selective loss in the IL of the cDKO (Figure 2.5F). Finally, ALPHA-CATENIN is similarly lost in the IL cells of the cDKO (Figure 2.5H), compared to the uniform expression seen in the control (Figure 2.5G). To investigate the impact of the loss of adherens junction proteins on the integrity of the posterior-intermediate lobe boundary, the location of the neuron terminals containing arginine vasopressin (AVP) was examined. AVP neurons (red) exclusively terminate in the PL of the control mice (Figure 2.5I) and are distinct from the POMC cells (green) in the IL. In contrast, cDKO pituitaries contain AVP immunoreactive axon terminals (red) extending into the IL, intermixed with POMC cells, and occasional large POMC cells in the PL (Figure 2.5J). Mice were also analyzed at P90 and P180 and adhesion phenotypes were similar to P30 (data not shown). Also, no differences between male and female mice were observed. Numb, then, appears critical to maintain cell-cell adhesion and proper separation of the posterior lobe from intermediate lobe.

Numb affects pituitary progenitor cell organization

Given that Numb affects progenitor and differentiated cell distribution during neurogenesis, and that we see reduced membrane localization of adhesion molecules in the cDKO pituitary, we investigated the distribution of Sox2 positive stem-like progenitor cells in the intermediate lobe (46, 76). Sox2 progenitor cells in the pituitary can also be E-cadherin positive (76) so we speculated that dysregulated E-CADHERIN in the cDKO pituitaries also affects the progenitor cell niche. It has been recently demonstrated that Sox2 positive cells in the intermediate lobe are found at the cleft border (46). We confirm this result in control animals by Sox2 immunostaining (Figure 2.6A), however in cDKO intermediate lobes, Sox2 positive cells are often observed throughout the intermediate lobe rather than confined to the lumen border (Figure 2.6B, arrowheads). The level of *Sox2* mRNA from the whole pituitary, however, appears unchanged (Figure 2.6, graph). Finding disorganized progenitor cells and improper E-CADHERIN expression, it was logical to speculate the niche of the Sox2 containing progenitor cells is disrupted, which might have downstream consequences on aspects of their behavior, such as proliferation. At P30, Ki67 positive cells are scattered throughout the intermediate lobe, not confined to

the Sox2 positive cells, and are approximately equal in location and number between control and cDKO animals (Figure 2.6C, D, graph). These data indicate that although *Numb* is necessary for intermediate lobe architecture and cell placement, cell proliferation is not controlled by these elements.

Numb actions on Notch activity

One of the best-characterized actions of Numb is to inhibit Notch signaling, therefore we questioned whether Numb normally suppresses Notch receptor actions and expression of downstream Notch targets in the pituitary. Notch2 is important for early pituitary development, yet becomes undetectable after embryonic day 14.5(42, 43). Therefore, Numb expression in the adult intermediate lobe may act in part to reduce expression of Notch receptors. We found no differences in *Notch2* or downstream targets *Hes1, HeyL, Hey1 and Hey2* mRNA between control and cDKO pituitaries isolated at P30 (Figure 2.8), or by immunostaining for NOTCH2 in POMC expressing cells (data not shown). Numb, then, does not appear to regulate Notch activity in the differentiated melanotropes.

Discussion

Numb is a complex evolutionarily conserved adaptor protein with important actions during embryonic development. This includes facilitating asymmetric cell division, cell adhesion and migration, endocytosis, and ubiquitination (13, 16, 22, 30, 33, 34, 79, 80). Numb does this through interactions with several pathways including Notch, Hedgehog, cadherins and p53(13, 30, 33, 34, 36, 37, 40, 80-82). Although the function of Numb during embryogenesis in other tissues has been well characterized, its role in differentiated cells is less well understood. The present study represents, to the best of our knowledge, the first characterization of NUMB expression and function in the pituitary. In the postnatal pituitary, we observe NUMB staining in gonadotropes as well as sporadically in the intermediate lobe. We show that loss of NUMB in the mouse intermediate lobe melanotropes dramatically alters cell adhesion, progenitor cell localization and results in posterior-intermediate lobe cell intermixing.

NUMB contains multiple protein interaction domains, allowing for a diverse range of functions. A domain that mediates many actions of NUMB is the phosphotyrosine binding (PTB) region. This area is best known to be critical for inducing Notch degradation, although it is also involved with localizing NUMB to the plasma membrane and interactions with integrin and cadherin proteins (8, 13, 30, 34, 80). The PTB region can be subject to alternative splicing and the longer form is required for Itch-mediated ubiquitination and endosome trafficking of NOTCH1 for degradation(13, 30). We find strongest expression of the longer isoform (PTB_L) before and at birth, with a decline, but not complete loss, into adulthood. It is known that the Notch signaling pathway is critical for early pituitary formation (42, 43, 67), but becomes largely undetectable after e14.5, and can be found in isolated pituitary stem cells (46, 73, 74). Although Numb and Notch signaling components appear to be expressed in the same location of the developing pituitary, it is unknown if Numb affects Notch signaling at that time. It may be that Numb controls two distinct but related functions of Notch during embryogenesis and adulthood via PTB_L: hormone cell specification and stem cell maintenance respectively. Based on our data, if the PTB is important for modulating Notch activity and transcriptional activation in adult pituitaries, it is likely occurring in a population of cells not expressing POMC. However, we cannot exclude the possibility of other unknown proteins restoring Notch activity and compensating for the loss of *Numb*, or of changes in RNA expression undetectable by our whole pituitary RTPCR analysis.

As previously mentioned, the PTB region is not only involved with NOTCH degradation, but can also bind cadherins (80). Following deletion of *Numb* in the intermediate lobe, we find altered E-CADHERIN with a loss of additional adhesion molecules including N-CADHERIN, BETA-CATENIN and ALPHA-CATENIN. Based on our isoform analysis, it is possible the residual PTB_L form detectable in adulthood is required for proper localization of these adhesion molecules. Likely as a result of these disrupted adhesion proteins, cellular organization in these cDKO intermediate lobes is highly disordered and the cells are significantly less dense. This corroborates with previous studies showing Numb is required for proper cadherin protein function and localization (33, 80). Strikingly, cDKO pituitaries show extensive mixing of POMC cells with posterior lobe projections expressing AVP, and many large irregularly shaped

POMC cells are evident in the posterior lobe region. One possible explanation might be some posterior lobe axon terminals receive improper guidance cues from pituicytes, which could be mislocalized to the intermediate lobe of cDKO mice. In another study, mice deficient in a protein necessary for docking and fusion of secretory vesicles, *Munc18-1*, caused initial establishment of the hypothalamo-neurohypophysial system, however before birth posterior lobe axon terminals failed to reach their proper targets(91). This demonstrates that communication between posterior lobe axon terminals and supporting glial pituicytes is necessary for proper gland formation (91). These results all suggest Numb is important for maintaining cell adhesion and pituitary morphology and it may have a similar function in other endocrine tissues in which it is expressed, such as the pancreas.

Another region of NUMB that mediates distinct interactions and is subject to alternative splicing is the proline rich region (PRR). We find PRR_L is most prevalent before and shortly after birth with a decline at P9, and it is detectable at low levels in the adult pituitary. Previous studies show longer isoforms of PRR occur predominantly in progenitor cells, and this expression profile has been supported by experiments in mouse pancreas, testes, and neurons (3-5). It is not well understood why PRR is preferentially localized to progenitor cells, or how it may promote the maintenance of these cells. However, experiments in neuronal progenitors show that *Numb* knockdown followed by re-expression of PRR_L isoforms in embryonic cortical progenitors at e13.5 and from P19 neurons caused an increased number of neuronal progenitors expressing Nestin, and these effects are Notch-independent (3). Pituitary progenitor cell maintenance and early expansion then may be mediated by PRR_L isoforms, and continued expression into adulthood may be related to preservation of the small population of Sox2 and Sox9 positive adult pituitary stem-like cells (46). Recently, it has been shown these Sox2 positive cells in the adult pituitary can differentiate into several hormone producing cells in culture, and are likely important for proliferating and upregulating hormone producing cells during times of physiological stress (46, 75, 76). We found a disruption in the distribution of Sox2 positive cells, but analysis of whole pituitary samples showed no difference in the amount of *Sox2* transcription. Numb is then important for maintaining the proper Sox2 positive cell niche. This aberrant localization of Sox2 positive cells could

be due in part to loss of important Numb-protein interactions in melanotropes, or from indirect effects of adhesion impairment by loss of adherens junction proteins.

One prediction, based on the known functions of Numb, is that intermediate lobe hyperplasia or tumors would result when *Numb* and *Numblike* are lost. The melanotropes are a relatively defined population and serve as a good model to investigate tumor formation because many mouse models lacking tumor suppressors are susceptible to intermediate lobe tumors (92-98). Recent studies have shown loss of NUMB has been implicated in invasive cancers (37, 99). Also there is evidence that Numb normally inhibits cell cycle progression through promoting p53 stability (36) and mutations in p53 are found in approximately 50% of all human malignant tumors (100). Given the role of *Numb* in maintenance of cellular quiescence in other tissues, we looked for differences in proliferation in the cDKO pituitaries. Proliferation, marked by Ki67, is unchanged between control and cDKO pituitaries. Additionally, no tumor formation is found, at least until 6 months of age. Although we did not examine p53 expression, it is known that loss of p53 in mice does not cause pituitary tumors, however loss of one or two copies of p53 can enhance tumor development in mice also heterozygous for the *Retinoblastoma* gene (*Rb*+/-) (101). Although proliferation remains the same between control and cDKO mice, the altered cadherin-catenin protein complex and mislocalized progenitor cells in the intermediate lobe parallels the phenotype of loss of Numb in dorsal forebrain radial glial cells. In these mice, neocortex rosettes containing progenitor cells, resemble primitive neuroectodermal human brain tumors (33). This suggests loss of Numb may not affect proliferation directly, but can induce clusters of progenitor cells, which may have an increased tendency to proliferate abnormally. The abnormal groupings of *Sox2* progenitor cells observed in the intermediate lobes of cDKO mice may have an increased likelihood of becoming cancerous if subject to an additional mutation or environmental insult according to the “hit” hypothesis in cancer development.

This work identifies NUMB and NUMBLIKE as putative regulatory proteins during early pituitary development as well as having critical functions in adulthood in the intermediate lobe. The actions of Numb and Numblike are broad, however we show these proteins are major components of proper cell adhesion in the pituitary intermediate lobe. NUMB expression in gonadotropes is intriguing, and future studies will attempt to

address if the function is unique or similar to that in the intermediate lobe. Given the evidence supporting a role of Notch proteins in cancer and pituitary adult stem cell maintenance, this highlights the importance of future studies to understand mechanisms by which Numb contributes to normal development and disease in other cell populations of the pituitary.

Materials and Methods

Mice

CD1 mice from embryonic day 9.5 to 12.5 (e9.5-e12.5), as well as adult pituitaries, were fixed in 3.8% formaldehyde for 1-24 hours, dehydrated in ethanol and then embedded in paraffin. Sagittal sections of 6 μm were mounted on charged slides and prepared for *in situ* hybridization or immunohistochemistry.

Pomc Cre mice and *Numb* and *Numblike* floxed mice were purchased from Jackson Laboratories (18, 83). A breeding colony was established with mice that contained both alleles of *Numb* and *Numblike* floxed (*Numb*fl/fl, *Numblike*fl/fl). These mice were bred to mice that had both *Numb* and *Numblike* floxed, and also contained the *Pomc* Cre transgene (*Pomc* Cre Tg; *Numb*fl/fl, *Numblike*fl/fl). *Numb* and *Numblike* floxed as well as *Pomc* Cre mice were genotyped according to previously published protocols (83, 102). All mice were maintained according to the University of Illinois IACUC.

In situ Hybridization

A sense and anti-sense probe for *Numb* were prepared from the pCMV-SPORT6 Vector containing a full-length mouse *Numb* cDNA (MGI: 3991630, purchased from Open Biosystems). The probe was linearized and then transcribed with T7 polymerase in the presence of digoxigenin labeled nucleotides to create an anti-sense probe and SP6 polymerase to create the sense probe. The slides were rehydrated with xylene, followed by a gradient of ethanol before equilibrating in phosphate buffered saline (PBS). After washing in PBS, the slides were then acetylated, after which a 1:1 solution of 2X hybridization solution (Sigma) and deionized formamide was put on each slide and the slides were incubated at 57°C. The probes were then denatured for 3 min. and the put on the slides under a coverslip overnight at 57°C. For the second day the slides were put in a

50% formamide 0.5 X SSC solution at hybridization temperature. The slides were then blocked with 10% heat inactivated sheep serum in Tris buffered saline (TBS) containing 2% bovine serum albumin (BSA) and 0.1% Triton X-100, followed by the application of the anti digoxigenin antibody conjugated to alkaline phosphatase (Roche). NBT-BCIP was added overnight for detection. Controls that were used included one section that no probe was applied to, a section with sense probe, and one with a *Mash1* probe that showed a consistent level and pattern of staining as previously described (72).

Immunohistochemistry

Antibodies used in the studies include: goat polyclonal NUMB antibody (1:3000, AbCam), rabbit polyclonal numb antibody (1:200, Upstate), rabbit polyclonal NUMBLIKE antibody (1:100, Proteintech Group), rat monoclonal Ki67 (1:100, Dako), rabbit polyclonal E-cadherin (1:150, Cell Signaling Technologies), mouse monoclonal N-cadherin (1:300, Zymed [Invitrogen]), rabbit polyclonal Beta-catenin (1:200, Cell Signaling Technologies) rabbit polyclonal Alpha-catenin (1:100, Santa Cruz), rabbit polyclonal arginine vasopressin (1:1000, Millipore), rabbit polyclonal Sox2 (1:400, Millipore). Hormone antibodies used include rabbit polyclonal GH, LH, PRL, TSH (1:1000, National Hormone and Peptide Program, NIDDK, Dr. A Parlow) and POMC (1:3000, DAKO).

To detect protein localization, slides were deparaffinized in xylene and rehydrated in a gradient of ethanol before equilibrating in PBS. For Ki67, E-cadherin, N-cadherin, Beta-catenin, Alpha-catenin, Sox2 staining procedures, slides were boiled in 0.01 M citric acid for 5-10 min. then allowed to cool for 10 min. All slides were then blocked in a suppressor serum of PBS containing 3% BSA, 0.1% Triton X-100 (IHCB), and 5% normal donkey serum. Then slides were incubated overnight at 4°C with the primary antibody diluted in IHCB. Primary antibodies were detected with either direct Cy3 or DyLight 488 conjugated (1:300) or biotin conjugated secondary antibodies, biotin-rabbit (1:250), biotin-goat (1:400), biotin-rat (1:250), biotin-mouse (1:200) and then amplified with either Streptavidin Cy3 or Strepavidin DyLight 488 (1:250). All secondary antibodies were obtained from Jackson Immunoresearch. During staining, slides are washed in PBS containing 0.05% Tween-20 and mounted in an aqueous florescence mounting media. These experiments were completed a minimum of three times on

independent samples, with 2 sections per sample being examined. Controls for single channel labeling involved running a sample in the absence of primary antibody. Controls for the rabbit double labeling included samples without each primary as well as samples lacking both primary antibodies, with a one hour blocking period between primary and detection antibodies using Donkey anti-rabbit IgG (1:100 Jackson Immunoresearch).

Experiments were viewed at 200x or 400x magnification with a Lecia DM2500 microscope and photographed with the Retiga 2000R color camera. The pictures were acquired in Q- Capture Pro after which they were transferred to Adobe Photoshop. Quantification of immuno-positive cells was recorded manually relative to total number of nuclei (DAPI) present in a complete 400x image intermediate lobe. For cell density quantification, total number of nuclei present in a 400x image of intermediate lobe section was compared to area determined using NIH ImageJ. Statistics were performed using Statistical Analysis Software (SAS).

Reverse Transcription PCR

Manually dissected pituitaries were put in RNAlater (Ambion) and stored at -20 degrees Celsius. Pituitaries were then moved to lysis solution and, using a homogenizer, were mechanically dissociated. An RNAqeous kit (Ambion) was then used to isolate the RNA from the homogenized samples. Reverse transcription was then performed to create cDNA from 0.5µg of isolated mRNA template. Following the formation of cDNA a PCR reaction was performed with primers for either PRR or PTB isoform of *Numb*. The PRR primers were: *Numb* RT-PRR Forward- 5'-CTT GTG TTC CCA GAT CAC CAG-3'. *Numb* RT-PRR Reverse- 5'-CCG CAC ACT CTT TGA CAC TTC-3'. *Numb* RT-PTB Forward- 5'-ATG AGC AAG CAG TGT TGT CCT GG-3'. *Numb* RT-PTB Reverse- 5'-ACA GCC ATG AAA CAA TGA CAG-3' (Bani Yaghoub et al., 2007). The PCR conditions for both primer sets used were 92ºC for 3 min (1 cycle), 92ºC for 30sec, 55ºC for 30 sec, 72ºC for 30sec (34 cycles), and 72ºC for 10 min. PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

Real Time PCR

RNA and cDNA was prepared from whole pituitaries as described above and all pituitaries used for real time analysis came from mice at P27. Samples were run and

analyzed on Biorad iCycler IQ. Primer sequences were developed on Beacon Designer 7.0. Primer sequences are: *Sox2* Forward 5'-GGA GAA AGA AGA GGA GAG AG-3' *Sox2* Reverse 5'-CTG GCG GAG AAT AGT TGG-3'; *GAPDH* Forward 5'-GGT GAG GCC GGT GCT GAG TAT G-3' *GAPDH* Reverse 5'-GAC CCG TTT GGC TCC ACC CTT C-3'; *Notch2* Forward 5'-TGC CAA TAC TCC ACC TCT C-3' *Notch2* Reverse 5'-TCC ACT GAC ACT GCT TCC-3'; *Hes1* Forward 5'- CTC GCT CAC TTC GGA CTC-3', *Hes1* Reverse 5'- GTG GGC TAG GGA CTT TAC G-3'; *HeyL* Forward 5'-GGA ACA ACA GAG AAT GAA C-3', *HeyL* Reverse 5'- CAG CAG TAG TGA GTA ACC-3'; *Hey1* Forward 5'-CAC GCC ACT ATG CTC AAT G-3', *Hey1* Reverse 5'-CCT TCA CCT CAC TGC TCT G-3'; *Hey2* Forward 5'-GAT TCC GAG AGT GCT TGA C-3', *Hey2* Reverse 5'-AGG TGC TGA GAT GAG AGA C-3'. The PCR conditions for all primer sets used were 95ºC for 20sec, 55ºC for 30 sec, 72ºC for 30sec (40 cycles).

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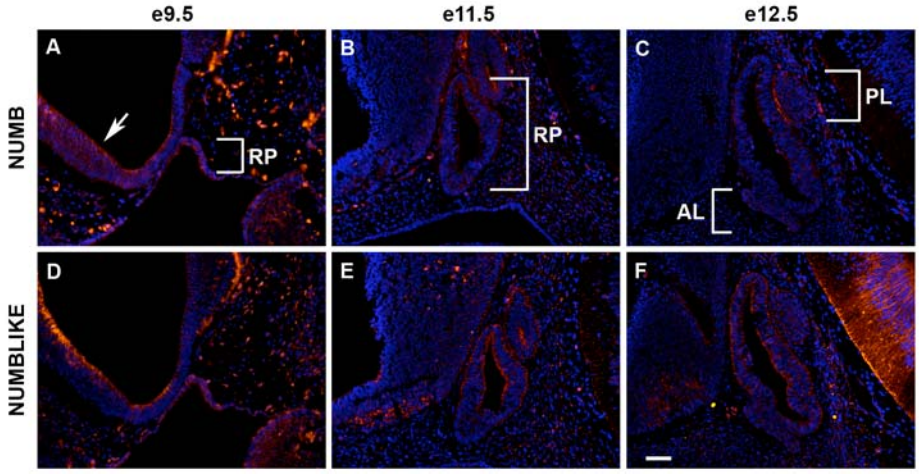
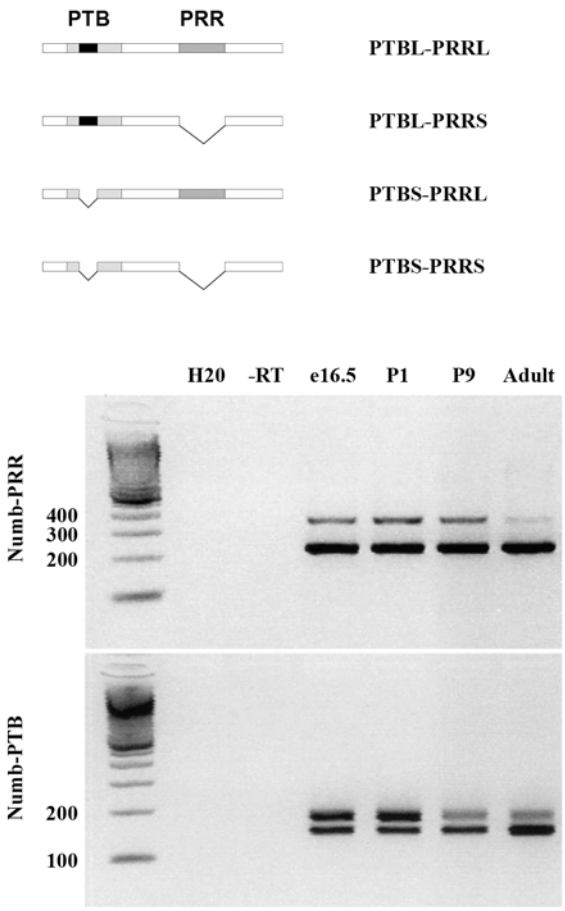
Figure 2.1



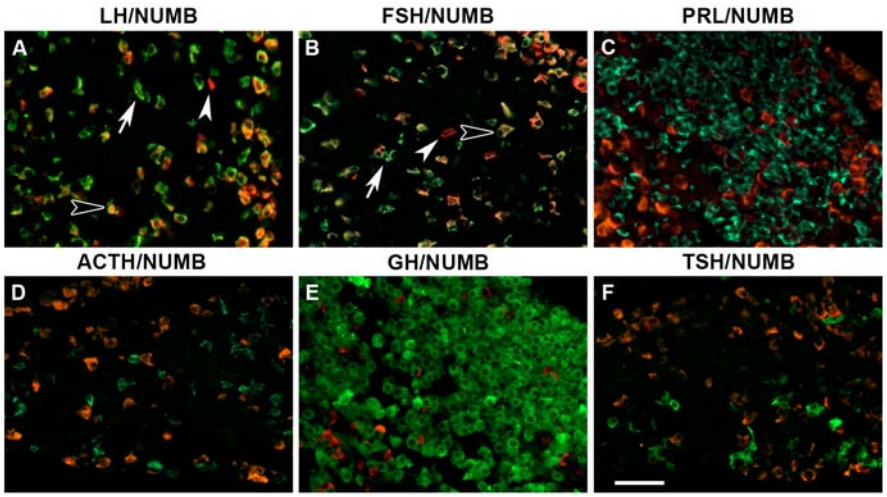
***Numb* and *Numblike* are preferentially expressed early in pituitary development.** Sagittal sections at e9.5 (A,D), e11.5 (B,E) and e12.5 (C,F) immunostained with Numb (A-C) and Numblike (D-F), counterstained with DAPI. Both Numb and Numblike are enriched in Rathke's Pouch (RP) at e9.5. AL = anterior lobe, PL = posterior lobe, arrow = ventricular zone. Pictures taken at 200x. Scale bar is 50 microns.

Figure 2.2



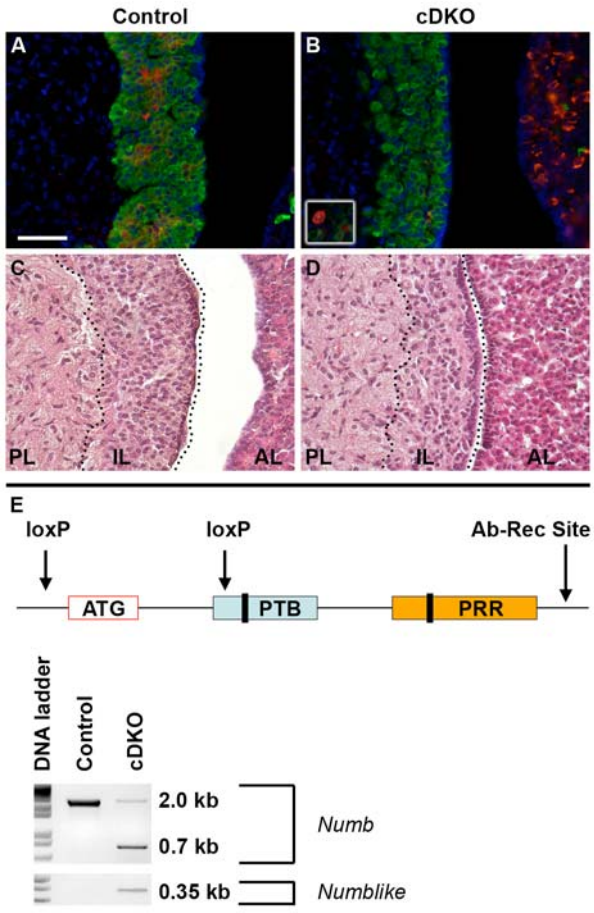
Shorter PRR and PTB Numb isoforms become dominant in adulthood. Diagram of alternate splicing isoforms for Numb. Reverse transcriptase PCR using primers spanning the proline rich region (PRR) and phosphotyrosine binding domain (PTB). Embryonic day 16.5 (e16.5), postnatal day 1 (P1), P9, and adult pituitary were analyzed. DNA standard measured in bp.

Figure 2.3



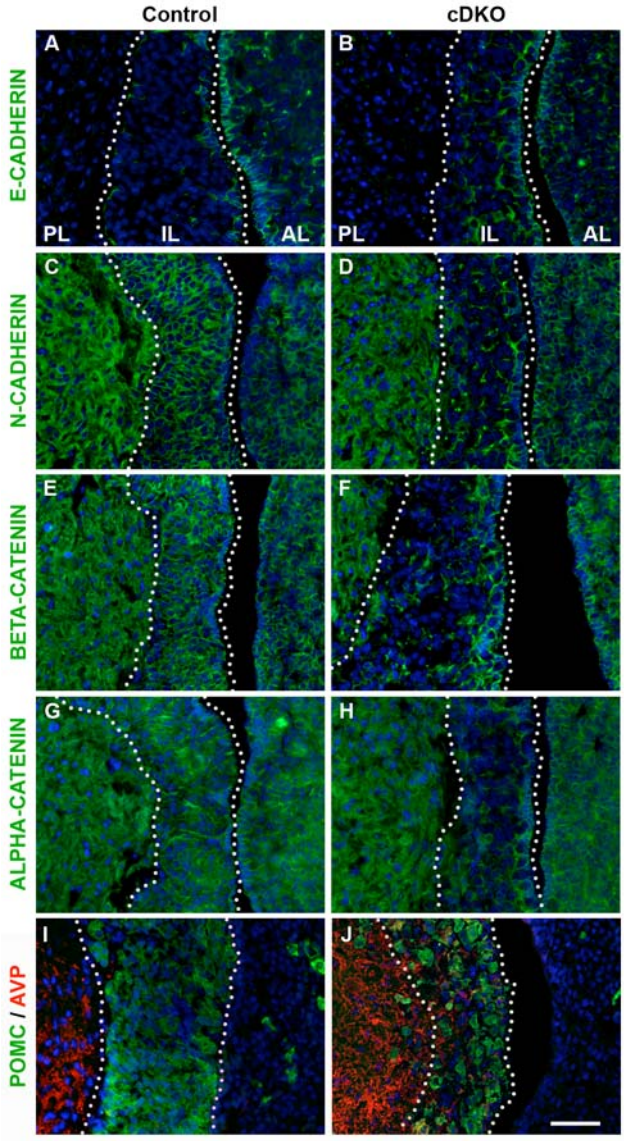
NUMB is expressed in gonadotropes in the anterior lobe. NUMB (red) is expressed in many but not all luteinizing hormone (LHβ, A) and follicle stimulating hormone (FSHβ, B) cells. NUMB is not expressed in prolactin (PRL, C), ACTH (POMC, D), growth hormone (GH, E), or thyroid stimulating hormone (TSHβ, F), producing cells. All hormones stained in green. Open arrowheads identify co-localizing cells, arrows show hormone positive, NUMB negative cells, while closed arrowheads show NUMB positive, hormone negative cells. Pictures taken at 400x. Scale bar is 50 microns.

Figure 2.4



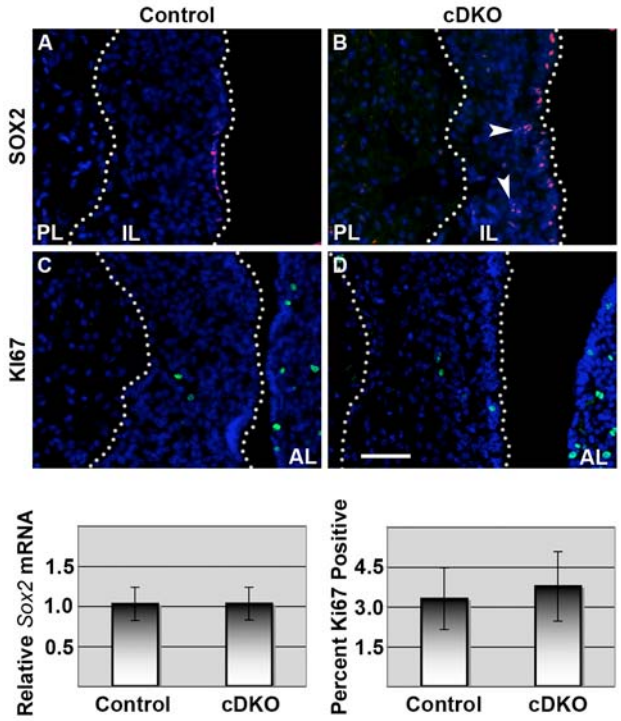
***Numb* deletion in intermediate lobe disrupts melanotropes.** NUMB immunostaining (red) shows positive cells in IL of control pituitary (A), and conditional knockout (cDKO) has reduced NUMB expression in IL (B) but expression in the AL is unaffected. Occasionally, large Numb positive cells are detectable in the cDKO intermediate lobe (white box, B). POMC immunoreactivity is similar between control (green, A) and cDKO (green, B), but occasionally POMC cells are seen in the PL of the mutant. Hematoxylin and eosin (H&E) stain showing distinct structures in the control pituitary (C). In the cDKO, cells in the IL (D) appear larger and disorganized. Schematic showing genomic deletion occurs around ATG start site (E). Deletion fragments of 0.7 and 0.35 kb correspond to *Numb* and *Numblike* respectively. Black bars within PTB and PRR regions represent alternative isoforms and antibodies used in this study recognize Numb C-terminal to PRR region. AL = anterior lobe, IL = intermediate lobe, PL = posterior lobe. Pictures taken at 400x. Scale bar is 50 microns.

Figure 2.5



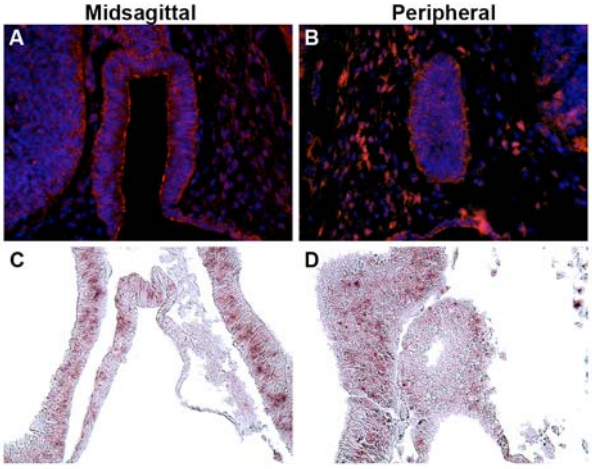
Conditional NUMB knockout in intermediate lobe disrupts adherens junctions protein expression and alters the posterior intermediate lobe border. E-CADHERIN immunostaining shows limited expression in control IL (A) but enhanced and highly disordered expression in the cDKO (B) intermediate lobes. N-CADHERIN (C,D), BETA-CATENIN (E,F), and ALPHA-E-CATENIN (G,H) all show disrupted expression in the cDKO (D,F,H) compared to control (C,E,G). Arginine vasopressin (AVP, red) positive axons located within cDKO IL (J) unlike the control (I). AL = anterior lobe, IL = intermediate lobe, PL = posterior lobe. Pictures taken at 400x. Scale bar is 50 microns.

Figure 2.6



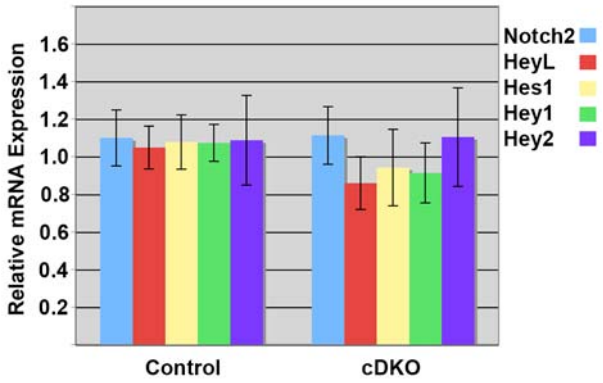
Numb affects pituitary progenitor cell organization but there is no change in progenitor cell number or proliferation. SOX2 immunostaining (red) in control pituitaries is limited to IL border (A) but is observed throughout cDKO IL (B). Ki67 immunostaining (green) in cDKO IL (D) does not differ visually compared to control (C). Real time PCR of whole pituitaries shows no *Sox2* mRNA differences between control and cDKO mice (n=4) after normalization to *Gapdh*. Cell counts of Ki67 positive cells compared to total number of IL cells shows proliferation is similar between control and cDKO (n=3). AL = anterior lobe, IL = intermediate lobe, PL = posterior lobe. Pictures taken at 400x. Scale bar is 50 microns.

Figure 2.7



NUMB is expressed in Rathke's Pouch at e10.5 by immunostaining and *in-situ* hybridization. Midsagittal sections (A,C) show sporadic Numb expression within Rathke's Pouch and along lumen border. Peripheral sections (B,D) shows a stronger outer border staining around Rathke's Pouch. Photographs taken at 400x.

Figure 2.8



***Notch2* and downstream target expression are not altered in Numb conditional knockout pituitaries.** Real Time PCR shows *Notch2* and downstream targets *HeyL*, *Hes1*, *Hey1*, *Hey2*, gene expression does not differ between control and cDKO whole pituitaries (n=4).

Chapter Three: Numb, an endocytic adaptor protein, is highly expressed in pituitary gonadotropes and may influence fertility

Abstract

The synthesis and release of the gonadotropins LH and FSH from the anterior pituitary are essential for fertility in males and females. Many cases of infertility that present with altered gonadotropin levels are idiopathic, making the identification of genes controlling gonadotrope development and function critically important. Notch signaling may be necessary for early gonadotrope lineage determination, however, it must be suppressed for timely hormone production in future gonadotropes. A candidate for suppressing Notch activity is the protein Numb, which causes degradation of the Notch receptor through polyubiquitination via interactions with the ubiquitin ligase Itch. Data from our lab show that Numb is preferentially localized in LH and FSH containing cells in the anterior lobe of the mouse pituitary. In the present study, we show by immunostaining that Numb is present in the rostral tip thyrotropes beginning at e13.5 and is detectable in αGSU expressing cells in the anterior lobe beginning at e15.5. To determine the function of Numb in the gonadotropes, Cre recombinase driven by the αGSU promoter, was used to delete Numb and its homolog Numblike (cDKO). At e15.5, Numb expression is reduced in cDKO pituitaries and adherens junction proteins are not altered. Finally, preliminary results indicate loss of Numb may affect both male and female fertility. Four of four male conditional Numb knockouts provided successful breeding, however two transgenic males not used for breeding had reduced testis size and low LH levels. Female Numb conditional knockouts produce litters, although with a significantly smaller number of pups. This study suggests *Numb* and *Numblike* may have a previously unidentified critical role in pituitary mediated reproduction.

Introduction

The mammalian pituitary is an important endocrine regulator of growth, metabolism, response to stress, and fertility. The adult anterior pituitary is composed of five hormone cell types, which include corticotropes, thyrotropes, somatotropes, lactotropes and gonadotropes. These cells are named after the hormones they secrete, and include adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone, (TSH), growth hormone (GH), prolactin (PRL), and follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively. The anterior and intermediate lobes of the pituitary develop from a common precursor primordium following an invagination of the oral ectoderm called Rathke's Pouch (RP). At approximately embryonic day 9.5 (e9.5), early transcriptional markers are detectable and important for formation of RP, and include LIM homeodomain transcription factors Lhx3, Lhx4 and Isl1, among others (59-61). Hormone production by these cells is largely temporally distinct, and the first detectable subunit mRNA expression includes alpha glycoprotein subunit (αGSU) at e12.5. This is a required subunit for TSHβ in thyrotropes and is also produced in TSH containing cells in the functionally distinct rostral tip thyrotropes. Expression of αGSU is also a critical subunit for both functional LH and FSH, produced in gonadotropes. Detectable levels of TSHβ occur around e14.5 and LHβ and FSHβ are detectable at e16.5 and e17.5 respectively (57, 103).

Gonadotrope lineage determination relies on a concert of extrinsic signaling pathways dictating expression of transcription factor gradients within the developing pituitary. The homeobox transcription factor *Pitx2* is one of the earliest induced genes that is necessary for gonadotrope specification. Mice with hypomorphic expression of PITX2 fail to activate transcription of other transcription factors important for gonadotrope function including *Gata2, Nr5a1 and Egr1* (104). The zinc-finger transcription factor GATA2, is a marker of early gondotrope differentiation and relies on BMP2 signaling, in addition to PITX2, for its expression. GATA2 has an antagonistic role with PIT1, a transcription factor necessary for the specification of thyrotropes, somatotropes and lactotropes. Specifically, in the absence of *Pit1*, more progenitor cells assume a gonadotrope fate, presumably due to enhanced GATA2 activity (105). Mice with a pituitary specific knockout of *Gata2* using the Cre-recombinase under control of

the αGSU promoter, have reduced gonadotropin levels, yet are fertile suggesting *Gata2* is dispensible for gonadotrope specification (106). Pituitary specific deletion of the nuclear receptor *Nr5a1* (steroidogenic factor 1 [SF1]) causes a complete lack of LHb and FSHb (107), while early growth response 1 (EGR1) is necessary for *Lhb* but not *Fshb* transcription (108). Additionally, the T-box transcription factor TBX19 normally acts to repress gonadotrope development, where loss of TBX19 leads to a conversion of intermediate lobe cells into gonadotropes, and overexpression of TBX19 suppresses the gonadotrope lineage (109). These factors play a role in proper expression of LH and FSH, however other factors clearly must be involved in determining early gonadotrope cell fate.

There are data to suggest that Notch signaling also plays a role in suppression of gonadotrope cell fate. NOTCH2 is expressed between e12.5 and e14.5 in the progenitor cells of RP but not in the differentiating hormone producing cells expressing αGSU (42), Further, persistent *Notch2* expression in gonadotropes causes a delay in their development (43). Finally, Notch signaling is a critical component of *Prop1* activation (67) and overexpression of PROP1 under control of the αGSU promoter causes reduced levels of *Lhb* and *Fshb* (110, 111). These results all suggest *Notch2* may be necessary for early gonadotrope lineage determination but must be extinguished before hormone production occurs in αGSU positive cells (43). A candidate for suppressing Notch activity is the protein NUMB, which causes degradation of the Notch receptor through interactions and subsequent polyubiquitination by the ubiquitin ligase ITCH (13, 30). Also, NUMB has recently been shown to be present in most, but not all, FSH and LH producing cells in the anterior lobe (Chapter 2). We hypothesized NUMB is required for gondadotrope differentiation, and maintenance into adulthood by suppressing NOTCH2.

Because *Numb* knockouts die at e11.5, prior to gonadotrope specification (17), we chose the αGSU Cre to conditionally delete *Numb* in αGSU expressing cells (112). This Cre will cause deletion of *Numb* in the gonadotropes and thyrotropes, although it should not affect thyrotrope development as we found no detectable NUMB expression in anterior lobe thyrotropes (Chapter 2). Previous groups have used the same αGSU Cre strain, and found pituitary knockout of floxed *Sf1* ($SF1^{fl/fl}$) results in severely hypoplastic testes and ovaries, concomitant with decreased levels of LH and FSH (107). Also, αGSU

Cre-dependent ERK knockout ($ERK2^{fl/fl}$ $ERK1^{-/-}$) results in reduced *Lhb* in females but normal levels in males. Interestingly, *Esr1* (ERα) knockout females have normal serum levels of LH and FSH, however have disrupted cyclicity and are infertile (113). Taken together, this evidence suggests a pituitary specific deletion of *Numb* using the αGSU-Cre is an appropriate model to understand the function of NUMB in gonadotropes.

Although NUMB plays a critical role in inhibiting Notch function, NUMB is also important for endocytosis and receptor trafficking (9, 30). Proteins such as Gli1, p53, and E-cadherin can be degraded or altered in expression through a NUMB mediated mechanism, and many other protein interactions with NUMB likely exist which have yet to be described. The regulation of gonadotropin expression in adults is complex and is influenced by pulsatile hypothalamic release of GnRH, and actions of many hormones including, activin, inhibin, follistatin, estrogen, and many others (114-116). It is possible that NUMB plays multiple roles in the pituitary and could influence LH and FSH expression or release independent of Notch signaling.

Although not complete, our studies show that Numb is highly expressed in gonadotropes at the onset of their differentiation. Very preliminary results show in the absence of Numb there may be fertility problems in females, while males are fertile but may have low expression of LH and reduced testis size. These results may or may not be related to Notch signaling, and have the potential to uncover a previously unidentified role of NUMB in gonadotropin receptor signaling and hormone expression.

Materials and Methods

Mice

CD1 mice from embryonic day 13.5 to 15.5 (e13.5-e15.5), as well as adult pituitaries, were immediately placed into RNAlater (Ambion) for RNA isolation, or fixed in 3.8% formaldehyde for 1-24 hours, dehydrated in ethanol and then embedded in paraffin. Sagittal sections of 6 μm were mounted on charged slides and prepared for immunohistochemistry.

αGSU-Cre mice were generously provided by the Camper Laboratory (University of Michigan) and *Numb* and *Numblike* floxed mice were purchased from Jackson

Laboratories (18, 83). A breeding colony was established with mice that contained both alleles of Numb and Numblike floxed ($Numb^{fl/fl}$, $Numblike^{fl/fl}$). These mice were bred to mice that had both Numb and Numblike floxed, and also contained the αGSU cre transgene (αGSU-Cre Tg; $Numb^{fl/fl}$, $Numblike^{fl/fl}$). *Numb* and *Numblike* floxed as well as αGSU Cre-Tg mice were genotyped according to previously published protocols (102, 112). All mice were maintained according to the University of Illinois IACUC.

Immunohistochemistry

Antibodies used in the studies include: rabbit polyclonal NUMB antibody (1:200, Upstate), rabbit polyclonal NUMBLIKE antibody (1:100 Proteintech Group), rabbit polyclonal E-cadherin (1:150 Cell Signaling Technologies), mouse monoclonal N-cadherin (1:300 from Zymed [Invitrogen]). Rabbit polyclonal αGSU (National Hormone and Peptide Program) antibody was used at 1:1500.

To detect protein localization, sildes were deparaffinized in xylene and rehydrated in a gradient of ethanol before equilibrating in PBS. For E-cadherin and N-cadherin staining procedures, slides were boiled in 0.01 M citric acid for 5-10 min. then allowed to cool for 10 min. All slides were then blocked in a suppressor serum of PBS containing 3% BSA, 0.1% Triton X-100 (IHCB), and 5% normal donkey serum. Then slides were incubated overnight with the primary antibody diluted in IHCB. Primary antibodies were all detected with biotin conjugated secondary antibodies, biotin-rabbit 1:250, biotin-mouse 1:200 and then amplified with either Streptavidin Cy3 or Streptavidin DyLight 488, both diluted 1:250. All secondary antibodies were obtained from Jackson Immunoresearch. During staining, slides are washed in PBS containing 0.05% Tween-20 and mounted in an aqueous florescence mounting media. These experiments were completed a minimum of three times on independent samples, with 2 sections per sample being examined. Controls for single channel labeling involved running a sample in the absence of primary antibody. Controls for the rabbit double labeling included a single primary antibody of each, and a sample with no primary antibodies, each of which included all secondary and tertiary antibodies.

Experiments were viewed at 200x or 400x magnification with a Lecia DM2500 microscope and photographed with the Retiga 2000R color camera. The pictures were acquired in Q- Capture Pro after which they were transferred to Adobe Photoshop.

Real Time PCR

Manually dissected pituitaries were put in RNAlater (Ambion) and stored at -20°C. Pituitaries were then moved to lysis solution and, using a homogenizer, the pituitaries were mechanically dissociated. An RNaqeous kit (Ambion) was used to isolate the RNA from the homogenized samples. Reverse transcription was then performed to create cDNA from 1 μg of isolated mRNA template. Samples were run and analyzed on Biorad iCycler IQ. Primer sequences were developed on Beacon Designer 7.0. Primer sequences are: LH Forward 5'-CCC AGT CTG CAT CAC CTT CAC-3' LH Reverse 5'-GAG GCA CAG GAG GCA AAG C -3';GAPDH Forward 5'-GGT GAG GCC GGT GCT GAG TAT G-3' GAPDH Reverse 5'-GAC CCG TTT GGC TCC ACC CTT C-3'. The PCR conditions for all primer sets used were 95°C for 20sec, 55°C for 30 sec, 72°C for 30sec (45 cycles).

Results

NUMB and NUMBLIKE are differentially expressed during late embryonic pituitary development

Previously, we showed by immunostaining that NUMB is highly expressed early in pituitary development, but becomes largely undetectable in RP by embryonic day 12.5 (e12.5) (Figure 2.1c). In these studies, we extended our embryonic analysis of NUMB expression and observe that it is almost exclusively present in the rostral tip thyrotropes (RTT) at e13.5 (Figure 3.1b). NUMBLIKE is expressed at low levels at e12.5 (Figure 3.1c), but becomes largely undetectable by immunostaining after e13.5 (Figure 3.1d). By e15.5 NUMB can be observed in the developing anterior lobe (Figure 3.1e) which are largely αGSU positive cells (Figure 3.1f). In postnatal day 21 (P21) pituitaries, Numblike RNA expression levels are only marginally higher than the negative control (no reverse transcriptase, data not shown).).

Conditional Deletion of NUMB with αGSU-Cre

NUMB co-localizes extensively with gonadotropins LHb and FSHb in the adult mouse anterior pituitary (Chapter 2), and is expressed in many αGSU positive cells in the RTT and anterior lobe at e15.5 before LHb and FSHb are detectable by immunostaining (Figure 3.1f). Both RTT cells as well as gonadotropes express αGSU, making the aGSU Cre an appropriate choice for conditionally deleting *Numb* and *Numblike*. In $Numb^{fl/fl}$, $Numblike^{fl/fl}$ e15.5 controls, NUMB is detectable in three independent embryos in the anterior lobe, as well as in RTT cells (Figure 3.2a-c). NUMB expression appears to be reduced in the AL of three independent $Numb^{fl/fl}$, $Numblike^{fl/fl}$ αGSU-Cre (cDKO) embryos (Figure 3.2d-f). NUMB does not appear to be dramatically reduced in the RTT of conditional knockout embryos, however one conditional knockout may lack NUMB expression there (Figure 3.2d).

Adhesion markers are not grossly affected by loss of Numb in embryonic pituitary

It has been demonstrated that NUMB is important for the maintenance of cell adhesion though interactions with E- and N-cadherin (33, 80) (our results, Chapter 2). At embryonic day 14.5, both E- and N-cadherin expression occur predominantly in the developing intermediate lobe with some low level expression in the anterior lobe (72) (Figure 3.3a,d). Preliminary immunostaining data (n=2) shows that E- (Figure 3.3b, c) and N-CADHERIN (Figure 3.3e,f) expression in cDKO embryos is not different compared to controls (Figure 3.3a,d).

***Numb* may affect gonadotrope function in males and females**

In the adult anterior lobe, NUMB is expressed in many but not all LHb and FSHb producing gonadotropes (Chapter 2). Given that NUMB levels are reduced in the cDKO pituitaries at e15.5, we have investigated the effects of loss of NUMB on fertility. Very preliminary results show two of three cDKO males at p21 have reduced *Lhb* mRNA levels (Figure 3.4 graph and chart). It is interesting to note these same two males had reduced testis size as well, and H&E stain shows no evidence of mature sperm (data not shown). Thus far four out of four cDKO males used in breeding pairs are fertile.

Similarly, female cDKO mice are fertile however so far they have reduced litter size to approximately half that of controls .

Discussion

The present study provides very preliminary yet fascinating evidence that NUMB has an important and previously unidentified role in mammalian fertility. We show that NUMB is largely expressed in αGSU positive cells in the developing anterior lobe during embryogenesis in early gonadotropes. When Numb is absent or reduced in gonadotropes, both male and female mice exhibit defects in the pituitary-gonadal axis. These results are even more intriguing when comparing the loss of function phenotype in the intermediate lobe of the pituitary. Conditionally deleting NUMB in the POMC expressing αMSH cells dramatically affected cell adhesion (Chapter 2). However, deleting NUMB in αGSU positive cells did not affect E- or N-CADHERIN expression or localization and provides additional support NUMB likely has a very different role in gonadotrope cells.

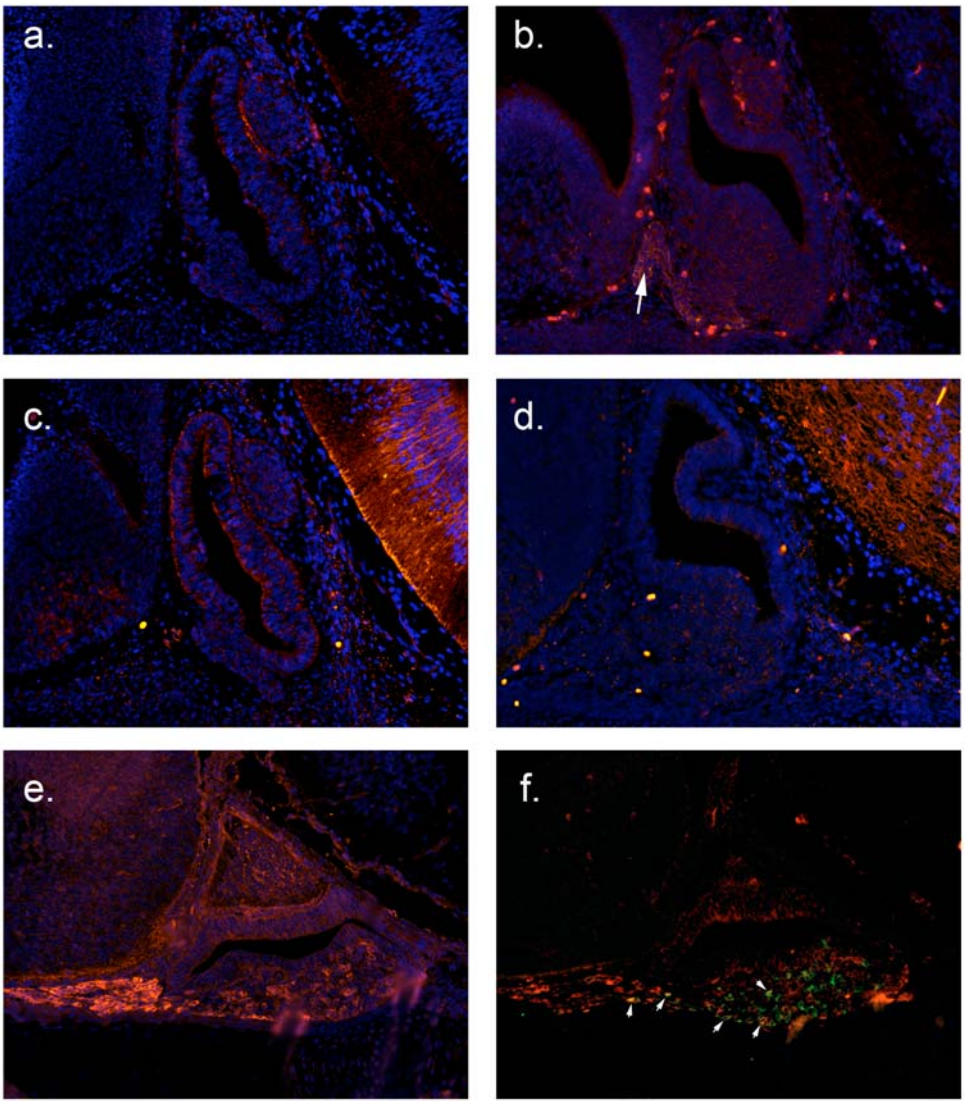
It is intriguing that NUMB is also strongly expressed in the poorly understood pars tuberalis (PT) or rostral tip thyrotropes. Recent evidence suggests the PT is involved with photoperiodic control of seasonal breeding. Increased secretion of TSH from the PT can alter medial basal hypothalamic control of GnRH and ultimately affect LH secretion and gonadal growth in the Japanese quail (117), and may alter seasonal breeding in mice as well (118). Given that NUMB is present in early gonadotropes as well as the PT and a subset of mature gonadotropes in the adult, it is tempting to speculate Numb may be involved in the circadian photoperiodic control of reproductive function.

There is increasing evidence Numb is critical for p53 stability, and dysregulated Numb is associated with human breast cancers (37, 99). However, an interesting link has developed suggesting estrogen activity may also be related to Numb function in breast cancers. Specifically, there is a significant correlation with higher levels of NUMB, and estrogen receptor (ER) and progesterone receptor (PR) expression in a subset of primary human breast tumor samples. Further, NUMB negative tumor samples are significantly correlated with the triple negative ER, PR and HER2 (human epidermal growth factor receptor 2) samples, suggesting a potential functional correlation between ER and PR, and Numb (119). NUMB is an endocytic adaptor protein, and may be necessary for

localization of proteins or membrane receptor recycling in mammary cells. The actions of the estrogen receptor also critically contribute to proliferation in the pituitary and is necessary for fertility. A recent conditional ERα knockout in the pituitary using the αGSU-Cre Tg, shows that LH and FSH serum levels are similar to those in control female mice, however these females have irregular estrous cycles and are infertile (113, 120). Estrogen is a critical mediator of cyclic LH release, and acts at the hypothalamic level to alter GnRH levels as well as directly in gonadotropes of the pituitary (121). Both ERα and ERβ are expressed in the anterior pituitary (122) however ERα is considered to be generally more important in pituitary control of fertility as ERα but not ERβ agonists induce LH secretion *in vitro* (123, 124), and $ER\alpha^{-/-}$ female mice cannot ovulate and are infertile, while $ER\beta^{-/-}$ females do ovulate and are subfertile (125, 126). Therefore NUMB and ERα may be involved with shared, or disparate pathways to regulate gonadotropin expression.

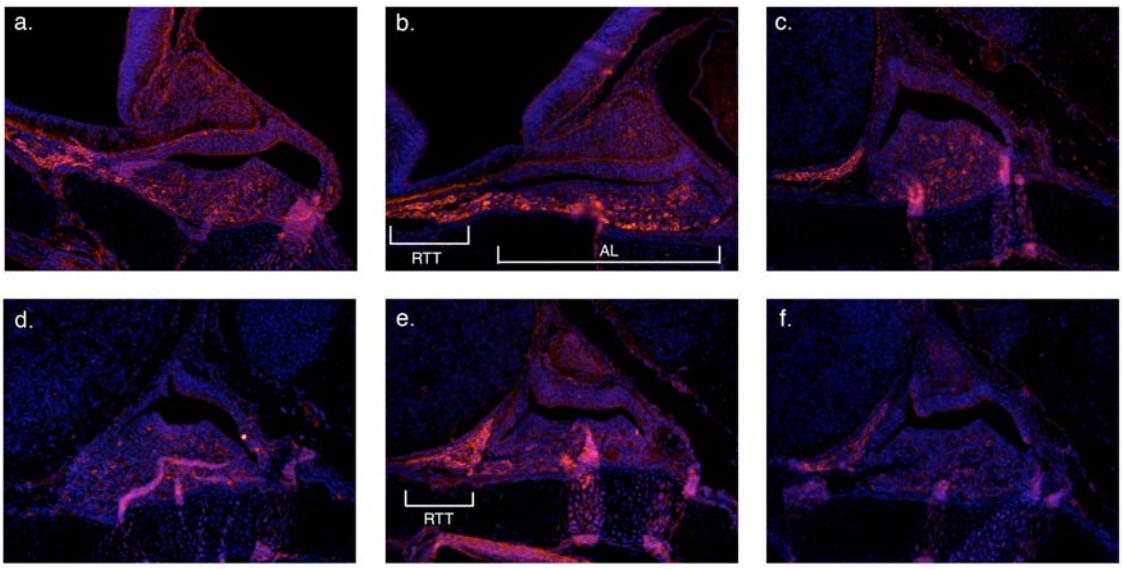
Our results, while preliminary, strongly support a role of NUMB in maintaining LH expression in males and possibly fertility in females. Given the emerging evidence of possible interactions between Numb and ER, we can speculate NUMB may have a previously unknown role in maintaining fertility, some of which could be through modulations of ERα activity. Well known for its nuclear activity, there are some reports of a cell membrane bound form of ER (127, 128). Therefore, as a membrane adaptor protein, it is possible NUMB could facilitate protein interactions with a membrane ER form. Also, NUMB has been reported to inhibit ubiquitin ligases, and so may help promote the stability of ER, or they may share common targets in a parallel pathway. For example, NUMB may interact with GnRH receptor (GnRHR), and like the Notch receptor, facilitate its recycling. Estrogen can influence GnRH release at the hypothalamic level, and therefore influence GnRHR activation. Finally *Notch2* expression has been reported in the adult pituitary (46), so NUMB may act to limit or control Notch activity in gonadotropes. Future experiments will investigate fecundity in cDKO mice as well as examine any tissue pathologies which may exist.

Figure 3.1



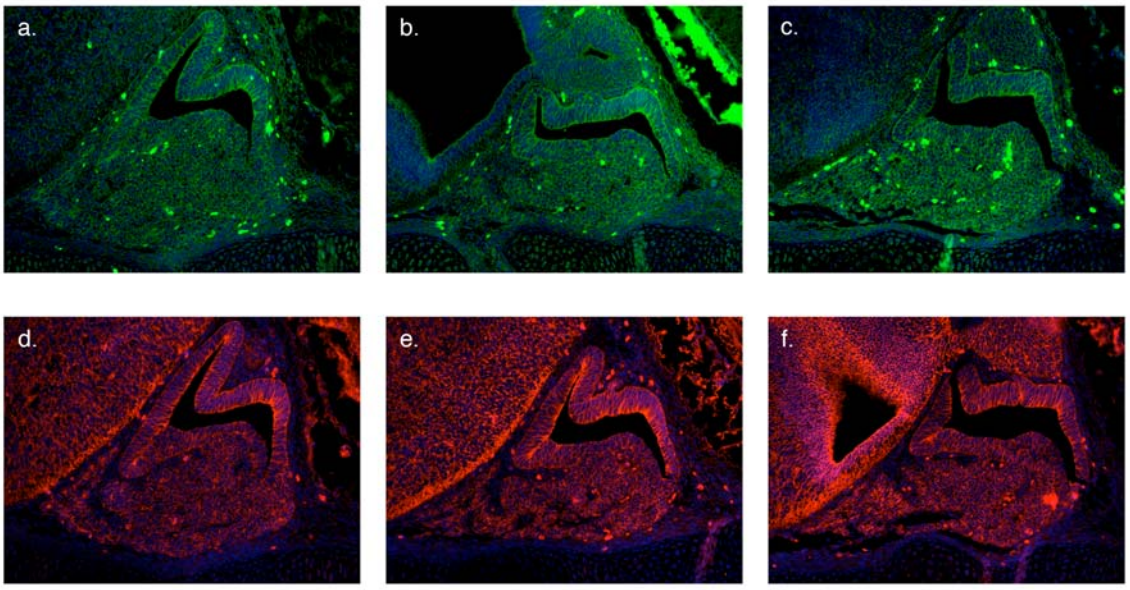
NUMB is expressed in RTT and αGSU positive cells later in development. Sagittal view of NUMB immunostaining at e12.5 (1a), NUMB expression is first evident in rostral tip thyrotropes (RTT), shown with arrow at e13.5 (1b). NUMBLIKE expression at e12.5 (1c) and e13.5 (1d). NUMB expression at e15.5 in RTT and scattered cells in anterior lobe (1e). Many NUMB positive anterior lobe cells (red) are also αGSU positive (green) shown with arrows at e15.5 (1f). Frames 1a, b, c, d photographed 400x, frames 1e, f photographed 200x.

Figure 3.2



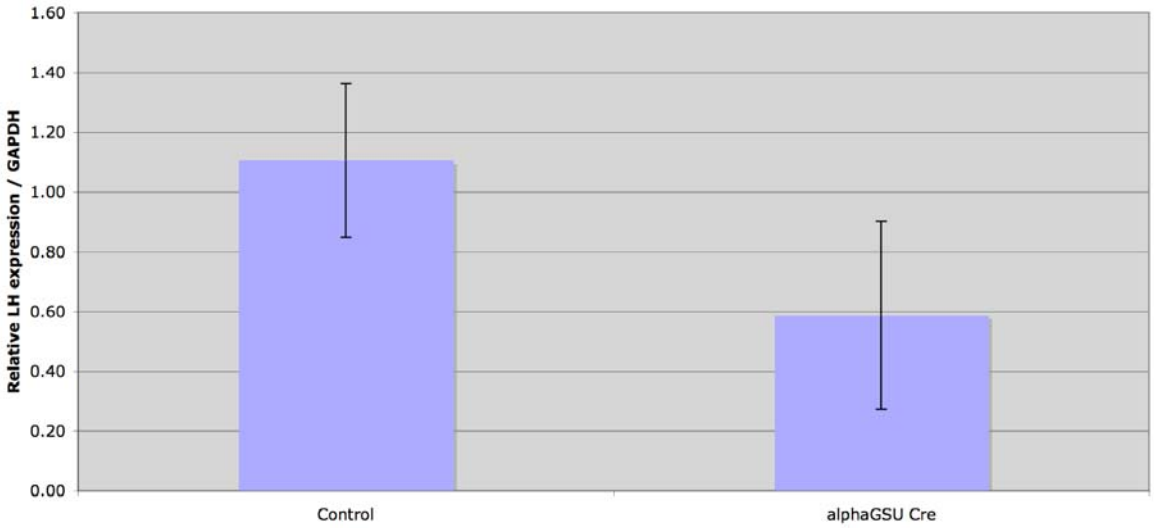
Loss of NUMB in αGSU Cre knockouts. NUMB staining in control e15.5 sagittal sections (2a, b, c) compared to conditional knockouts (2d, e, f). Three individuals of each genotype are presented. Loss of NUMB is observed in anterior lobe region, and variably in rostral tip thyrotropes (RTT). AL = anterior lobe. Pictures taken 200x.

Figure 3.3



E- and N-CADHERIN are unaffected by loss of NUMB in αGSU containing cells. E-CADHERIN (green) in two independent conditional *Numb* knockout pituitaries at e15.5 (3b, c) is indistinguishable from control (3a). N-CADHERIN (red) also appears normal in *Numb* knockout (3e, f) compared to control (3d).

Figure 3.4



Mouse Genotype	Relative LH Value	Phenotype
N fl/fl Nlk fl/fl	0.93	none
N fl/fl Nlk fl/fl	0.86	none
N fl/fl Nlk fl/fl	1.53	none
N fl/fl Nlk fl/fl +alphaGSUCre	1.10	none
N fl/fl Nlk fl/fl +alphaGSUCre	0.29	sm. testes
N fl/fl Nlk fl/fl +alphaGSUCre	0.37	sm. testes

Some evidence that LH may be reduced in NUMB conditional knockout males. Real time PCR showing individual relative LH values as calculated by standard delta delta Ct method. Two males with low LH levels relative to the three controls also had smaller testes.

Chapter Four: Aryl hydrocarbon receptor activation affects pituitary hormone cell function *in vivo* and *in vitro*.

Abstract

Studies investigating environmental contaminants such as dioxins have uncovered much about how these factors can disrupt endocrine function and contribute to cancer formation. Dioxins have been shown to activate the aryl hydrocarbon receptor (AhR), which acts as a transcriptional regulator at xenobiotic response elements in the genome. Understanding the effects of AhR activation has been of great interest to the reproductive biology community, however studies on mammalian hypothalamic pituitary axis function have been severely limited. In the present study, we examined the effect of AhR activation on endogenous pituitary hormone expression and proliferation in the GH3 rat somato-lactrotroph cell line. We show that nanomolar doses of the reversible AhR agonist β-naphthoflavone impairs mRNA transcription of PRL and GH, as well as protein expression of PRL in GH3 cells. Also, when combined with 100 nM of the partial AhR antagonist α-naphthoflavone, the suppressive effects on PRL were enhanced while GH levels were unchanged compared to control. These changes coincide with a suppression of the anti-proliferative signaling cytokine TGFbeta1. In knockout studies, we find that female $AhR^{-/-}$ mice do not have significantly different *Prl* or *Gh* levels, however there seems to be a trend towards increased *Gh* at postnatal day 90 (P90), with no change in *Prl*. However, $AhR^{-/-}$ mice do have significantly reduced *Lh* expression at P90. AhR interacting protein (AIP) has recently gained much attention following the identification of several mutations in a group of patients with pituitary adenomas in Northern Finland, and other groups have identified links between aberrant AhR activity and enhanced cell cycle progression. Surprisingly, we did not observe an alteration in cellular proliferation in GH3 cells treated in culture with beta-napthoflavone via flow cytometric analysis of propidium iodide staining. Overall, these results demonstrate that AhR is important for proper pituitary hormone cell development and maintenance, and represents a source by which environmental dioxins can exert endocrine disrupting effects.

Introduction

The aryl hydrocarbon receptor (AhR) is a basic-helix-loop-helix ligand activated transcription factor of the Per-ARNT-Sim (PAS) family, which regulates the response of many toxic aromatic hydrocarbons (129, 130). AhR ligands occur in common sources including pesticides and tobacco smoke (131). One of the more potent ligands includes 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a contaminant in an herbicide used in the Vietnam War. This dioxin is highly metabolically stable and causes several effects including thymic atrophy, chloracne, hepatomegaly, cachexia, teratogenic effects, and death in severe cases (reviewed in) (132). Activation of AhR mediated transcription occurs when a ligand binds inactive cytoplasmic AhR, which is normally associated with heat shock protein 90 (Hsp90) and immunophilin-like Ara9 or aryl hydrocarbon receptor interacting protein (AIP) (133, 134). This complex then translocates to the nucleus, dissociates, and the PAS domain of AhR forms a dimer with the PAS domain of the constitutively active nuclear protein aryl hydrocarbon receptor nuclear translocator (ARNT). At this point the AhR-ARNT complex binds xenobiotic or dioxin response elements (XRE, DRE) and mediates transcription (135, 136).

There is strong evidence to support a role for AhR in influencing endocrine function, particularly with respect to gonadal development and fertility (137-139). Female mice exposed to AhR ligands have a depletion of primordial and primary oocytes (140), and TCDD treatment causes reduced fecundity (141), cleft clitoris, and vaginal mesenchymal threads (142). Also, $AhR^{-/-}$ female mice have reduced conception, litter size, and pup survival (143, 144), and have impaired development of mature follicles (144-146). Data suggest disruptions in ovarian follicle development is an intrinsic effect, and mediated to some extent though interactions with estrogen, and exogenous estradiol administration can partially rescue the knockout phenotype (144). AhR can influence estrogen actions by directly binding to estrogen receptors and activating or inhibiting transcription of estrogen downstream target genes in a ligand and concentration-dependent manner. Specifically, in MCF-7 breast cancer cells, AhR-ARNT complexes associate with both ERα and ERβ in the absence of estrogen and activate transcription at estrogen response elements (ERE) with co-activator p300 (147). However when estrogen is bound to ER, AhR can inhibit ER-mediated DNA binding. AhR can also affect

androgen and progesterone receptor actions (148, 149), further expanding its role as an endocrine disruptor. Collectively, AhR is important for reproduction and additionally has a vast array of interactions to facilitate or inhibit steroid hormone actions.

While AhR can dramatically affect reproductive endocrine function, little is understood about how AhR regulates pituitary hormone synthesis and secretion. Environmentally relevant concentrations of TCDD cause reductions in serum LH and FSH levels in preovulatory rats, although it is unknown if the site of action is the hypothalamus or pituitary (52). Dioxin effects on the pituitary appear not limited to gonadotropins, as TCDD-treated mice have a three-fold increase in *POMC* mRNA levels, and the pituitary cell line AtT-20 show increases in *POMC* mRNA as well after TCDD treatment (53). More recently, two groups looking at rainbow trout pituitaries have found somewhat conflicting results. TCDD in nM concentrations causes increased *GH* and *PRL* mRNA *in vitro* (54). However, another study found decreased *GH* and *POMC* by microarray following feeding experiments of the much less potent AhR agonist β-naphthoflavone (55). These results show AhR can alter transcription of many pituitary hormones, and differences in magnitude and direction of gene changes may be due to ligand or concentration effects.

Additional evidence highlighting the importance of AhR in normal pituitary function came from the identification of germline mutations in AIP, as a cause of familial growth hormone secreting pituitary adenomas (51). Two AIP mutations accounted for 16% of all patients with growth hormone secreting tumors, and remarkably the age of diagnosis was significantly lower for patients with AIP mutations with 40% under age 35. Moreover, AIP expression is reduced in invasive versus non invasive somatotrophinomas (50), and AIP silencing in the somatolactotroph cell line GH3 causes increased proliferation (150). While not common in sporadic pituitary adenomas (151, 152), the aggressive nature and early onset of pituitary adenomas with AIP mutations clearly warrant further investigation on how AhR affects normal pituitary function and proliferation.

Alterations in AhR activity can cause serious health concerns, particularly in endocrine, reproductive and hepatic tissues. Studies on the direct actions of AhR in the pituitary are limited, however evidence suggests AhR ligands can alter normal pituitary

hormone production. Additionally, increased AhR activity, presumably through mutations in AIP, may be able to cause adenomas. The present study shows AhR activation can alter hormone production *in vitro* in the pituitary GH3 cell line, as well as *in vivo*, following loss of function in $AhR^{-/-}$ mice.

Materials and Methods

Mice

$AhR^{-/-}$ mice were generously provided and genotyped by B. Karman and M. I. Hernandez from the laboratory of Jodi Flaws, University of Illinois. Mice were originally developed from C. Bradfield's laboratory at the University of Wisconsin, but bred and housed for these experiments at the University of Illinois. Pituitaries from mice at postnatal day 3 and postnatal day 90 were dissected and stored in RNAlater (Ambion) at -20°C. All mice were maintained according to the University of Illinois IACUC.

Cell culture

GH3 cells were obtained from American Type Culture Collection (ATCC; Manassas, Virginia). Cells for RNA isolation were plated at 600,000 per well in 12-well plates and incubated in DMEM/F12 (1:1) media without phenol red (Hylcone) with 10% charcoal dextran treated fetal calf serum and 10 nM, 100 nM, or 1 μM β-naphthoflavone with or without 100 nM α-naphthoflavone for 20 hours. Cells for each condition were plated in triplicate. β-naphthoflavone (Sigma) and α-naphthoflavone (Sigma) were dissolved in DMSO and controls were treated in DMSO alone. The final concentration of DMSO in media in all conditions was 0.07%.

RNA isolation and QPCR

RNA isolation from cultured cells was performed by standard Trizol (Invitrogen) extraction. Pituitary RNA was isolated with an RNaqeous kit (Ambion) after tissue disruption in lysis solution with a homogenizer. cDNA synthesis with 0.5 μg of RNA was performed with Superscript II kit (Invitrogen). Samples were run and analyzed on Biorad iCycler IQ. Primer sequences were developed on Beacon Designer 7.0. Primer sequences

for $AhR^{-/-}$ mice: LH Forward 5'-CCC AGT CTG CAT CAC CTT CAC-3' LH Reverse 5'- GAG GCA CAG GAG GCA AAG C -3'; PRL Forward 5'-TCA GCC CAG AAA GCA GGG ACA-3'; PRL Reverse 5'- GGC AGT CAC CAG CGG AAC AGA -3'; GAPDH Forward 5'-GGT GAG GCC GGT GCT GAG TAT G-3' GAPDH Reverse 5'-GAC CCG TTT GGC TCC ACC CTT C-3'. Primer sequences for GH3 culture experiments: GH Forward 5'-AGG GCA TCC AGG CTC TGA T-3'; GH Reverse 3'-GCA TGT TGG CGT CAA ACT TG-3'; PRL Forward 5'-CAT CAA TGA CTG CCC CAC TTC-3'; PRL Reverse 5'-CCA AAC TGA GGA TCA GGT TCA AA-3'; ERbeta Forward 5'-TTG GTG TGA AGC AAG ATC ACT AGA G-3'; Beta-actin Forward 5'-AAC CCT AAG GCC AAC CGT GAA AAG-3'; Beta-actin Reverse 5'-CGA CCA GAG GCA TAC AGG GAC AAC-3'; TGFbeta1 Forward 5'-TCC AAA CGT CGA GGT GAC-3'; TGFbeta1 Reverse 5'-CAG GTG TTG AGC CCT TTC CA-3'; AhR Reverse 5'-TCG CGT CCT TCT TCA TCC GTT AGC-3'; AhR Forward 5'-TCA CTG CGC AGA ATC CCA CAT CC-3'; ERalpha Forward 5'-CCA AAG CCT CGG GAA TGG-5'; ERalpha Reverse 5'-AGC TGC GGG CGA TTG AG-3'; ERbeta Reverse 5'-AAC AGG GCA GGC ACA ACT G-3'; CYP1A1 Forward 5'-GTC CCG GAT GTG GCC CTT CTC AAA-3'; CYP1A1 Reverse 5'-TAA CTC TTC CCT GGA TGC CTT CAA-3'; The PCR conditions for all primer sets used were 95 degC for 20sec, 55 degC for 30 sec, 72degC for 30sec (45 cycles).

Data were analyzed according to standard delta delta CT method with n=3 for each experimental condition and graphed. Error bars show standard error of mean and statistical analysis was done using analysis of variance (ANOVA) using SAS.

Western Blot Analysis

Cells were plated as described above, however cells in both standard media (DMEM/F12 (1:1) with phenol red, 10% fetal calf serum) and charcoal dextran treated serum in DMEM/F12 (1:1) without phenol red were analyzed. Treatments included 10 nM, 100 nM, or 1 μM β-naphthoflavone and DMSO controls for 48 hours. Cells were then removed from plates with trypsin, washed twice in phosphate-buffered saline (PBS) and lysed in 400 mM NaCl RIPA buffer (Thermo Scientific) for 20 min. on ice with (1:100) mammalian protease inhibitor cocktail (Sigma). The lysate was centrifuged

(12,000 RPM, 15 min. at 4°C) and protein concentration of the supernatant was determined via Protein Assay (Biorad). Protein extracts were run on 4-20% SDS Page gels with 14 μg per well and transferred to nitrocellulose transfer membrane (GE). Blots were blocked in 5% milk 50 mM Tris pH 7.5 150 mM NaCl 0.5% Tween 20 (wash buffer) for 1 hour. Beta-tubulin rabbit polyclonal (Santa Cruz) was incubated overnight 4°C in wash buffer without milk at 1:2000. Rabbit polyclonal PRL (National Hormone and Peptide Program) used at 1:2000 was incubated in wash buffer the next day at room temperature for 1 hour. Secondary antibody for detection was goat anti-rabbit IRDye 800CW (Odyssey). Protein bands were recorded using an Odyssey LI-COR infrared scanner and band intensities were quantified via Odyssey 2.1 software.

Cell culture for cell cycle analysis

GH3 cells were plated at 250,000 cells/well in 12-well plates and serum starved for 3 days in 0.1% serum to synchronize cell cycles prior to 3 days of β naphthoflavone treatment in either standard media (DMEM/F12 (1:1) with phenol red) or with charcoal dextran stripped serum and phenol red free media. Cells were stained in propidium iodide after 3 days of β naphthoflavone treatment and analyzed by flow cytometry. Cell cycle analysis was performed by FCS Express. Graph represents n=3.

Results

β-naphthoflavone, and when combined with α-naphthoflavone, activates *CYP1A1*, suppresses *AhR*, and does not dramatically affect *ER* expression

Expression of CYP1A1 (cytochrome P4501A1) is the most well known and commonly used indicator of AhR activation *in vivo* and in culture (153-155). In fact, adult mouse pituitaries exhibit a robust induction of *Cyp1a1* following TCDD treatment (53). To determine if the somatolactotrope GH3 cells also respond to AhR activation, cells were treated with β-naphthoflavone and α-naphthoflavone and the levels of CYP1A1 were determined by real time RT-PCR. To reduce the actions of steroid hormones, which might alter AhR-mediated effects, select experiments for RTPCR used

charcoal dextran treated serum (Figures 4.1-3). Compared to vehicle treatment, β-naphthoflavone significantly increased *Cyp1a1* expression to levels approximately 6-fold higher than controls at the highest dose (Figure 4.1a). Interestingly, 100 nM of the partial antagonist α-naphthoflavone had no effect alone, but significantly enhanced the β-naphthoflavone-induced activation of CYP1A1 to levels approximately 14 fold higher than control samples. This shows α-naphthoflavone acts as an agonist in this context at 100 nM.

It has been reported AhR can upregulate its own expression in the pituitary in response to TCDD and β-naphthoflavone treatments (53, 55), and altered levels of AhR are important to consider when interpreting gene expression changes. In contrast to previously reported effects on AhR expression in the whole pituitary (53), we find that AhR activation causes suppression of *AhR* transcription in GH3 cells (Figure 4.1b). Strong suppression of *AhR* is evident in β-naphthoflavone plus α-naphthoflavone conditions, which correlate with the highest *CYP1A1* induction.

Estrogen signaling can have a profound effect on the expression of GH and PRL (156, 157). AhR has been reported to interact with both ERα and ERβ, recruit co-activator p300 to estrogen response elements and initiate transcription in human breast cancer-derived MCF-7 cells (147). Therefore, it is useful to determine if AhR is altering the expression levels of ER, and subsequently causing indirect effects due to altered ER-dependent transcriptional events. We find *Esr1* (ERα) expression levels in GH3 cells are significantly reduced at 100 nM of both β-naphthoflavone and α-naphthoflavone treatment, although the reduction is small (Figure 4.1c). *Esr2* (ERβ) is expressed at much lower levels than ERα, and those levels were not significantly different at any dose (data not shown).

Growth hormone and prolactin are differentially affected by AhR activation

Based on conflicting results of AhR activation on GH levels in trout pituitary cultures and its inductive effects on PRL expression (54, 55), we examined whether β-naphthoflavone with or without α-naphthoflavone, alters *Gh* or *Prl* mRNA expression in GH3 cells. Treatment of GH3 cells with β-naphthoflavone significantly reduced *Gh*

expression only with 100 nM β-naphthoflavone (Figure 4.2a). *Prl*, however, was significantly suppressed at all concentrations of β-naphthoflavone tested, with the greatest effects at 100 nM. Adding α-naphthoflavone enhanced the suppression of *Prl* to approximately half the expression of DMSO and DMSO plus α-naphthoflavone controls. This shows AhR activation can significantly reduce *Prl* but not *Gh* expression.

To determine if factors removed through charcoal dextran treatment might differentially affect *Gh* and *Prl* transcription compared to standard growth media, GH3 cells were treated with β-naphthoflavone and analyzed after 48 hours in each growth media (Figure 4.2b). *Gh* was not changed in any condition (data not shown), however *Prl* is significantly reduced following 1 μM β-naphthoflavone in standard, but not charcoal dextran treated media.

With the knowledge that AhR activation can suppress prolactin transcription, we performed western blot analysis to determine any changes at the protein level. Consistent with the 48 hour β-naphthoflavone mRNA data, β-naphthoflavone did not alter PRL in charcoal dextran treated media (Figure 4.2c). However, in standard media conditions, β-naphthoflavone did reduce PRL expression. Western blot band intensities were quantified and normalized to beta-tubulin controls using Odyssey 2.1 (LI-COR) software, showing that β-naphthoflavone at 1 μM reduced PRL to levels on average of 58.4 +/- 14% that of DMSO controls, n=3.

AhR suppresses TGFβ1

AhR activation is correlated with decreased expression levels of extracellular cytokine transforming growth factor β (TGFβ), and this may be important for AhR-mediated pathologies. For example, TCDD treatment causes reduced TGFβ1 expression in epithelial and mesenchymal cells during palatogenesis (158). Additionally, the absense of *Ahr*, in *Ahr*$^{-/-}$ mice leads to increased TGFβ levels in fibrotic livers (159). We show β-naphthoflavone at 100 nM can significantly reduce *Tgfb1* expression, and β-naphthoflavone plus α-naphthoflavone significantly reduces *Tgfb1* to a greater extent (Figure 4.3).

AhR knockout mice have reduced LH expression at P90

As a step toward understanding the physiological role of AhR in the pituitary, we examined expression of *Gh*, *Prl*, and *Lhb*, at post natal day 3 (P3), several days after the cells are specified, in mice with or without *Ahr*. We chose to focus on these hormones because of the significant effect loss of AhR has on ovarian physiology, as well as the effects on somatolactotropes described here and elsewhere (54, 55). Conflicting evidence exists concerning the effects of AhR on gonadotropin levels. TCDD can cause reductions in serum LH and FSH levels in preovulatory rats (52). However, in $Ahr^{-/-}$ mice, serum LH and FSH levels are relatively normal between ages P25 and P28 (160). We find that at P3 there is a trend towards reduction of *Lhb* ($p = 0.053$) and potentially a reduction of *Prl* in $AhR^{-/-}$ females (Figure 4.4a). At P90, there is a trend towards increased *Gh* while *Lhb* is significantly reduced in $Ahr^{-/-}$ females ($p < 0.001$) compared to wild type littermate controls (Figure 4.4b).

AhR activation by β-naphthoflavone does not alter cell cycle progression in GH3 cells

Specific mutations in AIP are associated with pituitary adenomas (51), where presumably AIP no longer sequesters AhR to the cytoplasm and allows enhanced AhR-mediated transcription. Further, AIP knockdown in GH3 cells enhances proliferation (150) and over expression of AhR enhances proliferation in carcinomic human alveolar basal epithelial A549 cells and mouse mammary fibroblasts (161, 162). Based on this proliferative effect of AhR activation, we investigated if β-naphthoflavone following three days of treatment enhances cell cycle progression by propidium idodide staining and flow cytometric analysis. Our data show that β-naphthoflavone at 1 and 10 μM did not alter the percentage of cells in G1, S, or G2 phases (Figure 4.5a,b). It is interesting to note that there appears to be a small difference between growth media, such that cells grown in the standard media tended to have fewer cells in G1 and more in S phase, which would be expected given the additional presence of steroid hormones. Overall, this suggests AhR activation at these levels is insufficient to alter proliferation in either media growth condition.

Discussion

The work presented here demonstrates that AhR activity affects pituitary hormone production *in vitro* as well as *in vivo.* This was first shown by β-naphthoflavone effectively inducing AhR activation through CYP1A1 expression in GH3 cells, which was expected based on previous studies *in vivo* and *in vitro* (55, 163). Unexpectedly, the AhR antagonist α-naphthoflavone had a synergistic effect with β-naphthoflavone in inducing CYP1A1, yet had no effect on CYP1A1 alone with the same concentration. The actions of α-naphthoflavone here are contradictory to previous reports of CYP1A1 induction in GH3 cells (164), however there are several reasons that could account for this. The study from Gauger et al. (2007) used PCB congeners for AhR induction and was antagonized with α-naphthoflavone, so the differences observed here from β-naphthoflavone could be due to different effects between the AhR agonists and α-naphthoflavone. It is possible α-naphthoflavone in the presence of different agonists may alter AhR conformation and subsequent transcriptional activity. Further, the concentrations of α-naphthoflavone used in our study of 100 nM was lower than the 1 μM levels used by Gauger et al. (2007), therefore in our experimental conditions and drug concentrations, α-naphthoflavone exhibited agonist actions. The GH3 cells described here for RTPCR data were cultured in media with serum that had been treated with charcoal dextran, which depletes the media of steroid hormones. Other groups have used alternate methods of removing steroid hormones including an anion exchange column (AG 1-X8 resin), which reduces T_3 and T_4 levels (164). Therefore it is possible different ligands and concentrations of steroid hormones were present during culturing conditions between studies. Last, treatments in this study examining mRNA levels were acute and consisted of 20 hours, which could have very different effects than studies with several day treatments.

Robust AhR activation, identified by greatest CYP1A1 induction, occurred in the cells treated with 1 μM β-naphthoflavone and 100 nM α-naphthoflavone. These same conditions caused the most *Prl* gene suppression, yet had very modest effects on *Gh* expression. Further, while a difference in PRL protein expression was not detectable following 48 hours of β-naphthoflavone treatment in charcoal dextran treated serum media, there was an observed suppression of PRL in standard media growth conditions.

These results appear to contrast with the reported findings of Elango et al. (2006) in which TCDD increases both *GH* and *PRL in vitro*, however at very low doses in the picomolar range, TCDD may suppress both GH and PRL (54). Also TCDD is a much more potent ligand than β-naphthoflavone, and therefore, these differences may be dose and ligand specific and highlights the importance of understanding "mixture effects." This concept of multiple ligands causing an outcome which is different than the sum of each of the independent actions was identified as an important consideration for future studies in a recent statement from the Endocrine Society (165). Each study, however, provides important insight that AhR can disrupt pituitary hormone synthesis, and the differences we observe in PRL but not GH expression may be due to variants in xenobiotic response elements for each gene.

These data identifying a pituitary hormone suppressing effect of AhR activation are complemented by the results of our knockout study. While not significant, there was a trend towards increased *Gh* at P90 in *Ahr*$^{-/-}$ animals. It is interesting to note that this effect mirrors to some extent, the significantly decreased *Gh* mRNA following 100 nM β-naphthoflavone treatment in the GH3 cells. This suggests AhR may normally act to subtly modulate, and specifically suppress, GH levels in adult animals. At P3, AhR$^{-/-}$ females have reduced *LH* levels, though not statistically significant. However, by P90 *Lh* is suppressed to a significant degree. This not only shows AhR has an important role in normal reproductive endocrine function, but may also partially explain the phenotype of impaired antral follicle development and reduced corpora lutea in *Ahr*$^{-/-}$ females (145, 146). Despite the fact that these mice have normal LH serum levels between P25 and P28 (160), there may be a progressive pre-pubertal suppression of LH which alters ovarian follicle development, before LH levels can be later restored. Further evidence to support a role in AhR directing *Lh* transcription came from studies showing fetal rats had reduced *Lh* mRNA as well as LH serum protein levels following maternal administration of TCDD (166, 167). Additional studies to follow *Lh* mRNA levels in *Ahr*$^{-/-}$ females during puberty and into adulthood could be illuminating on understanding this ovarian phenotype.

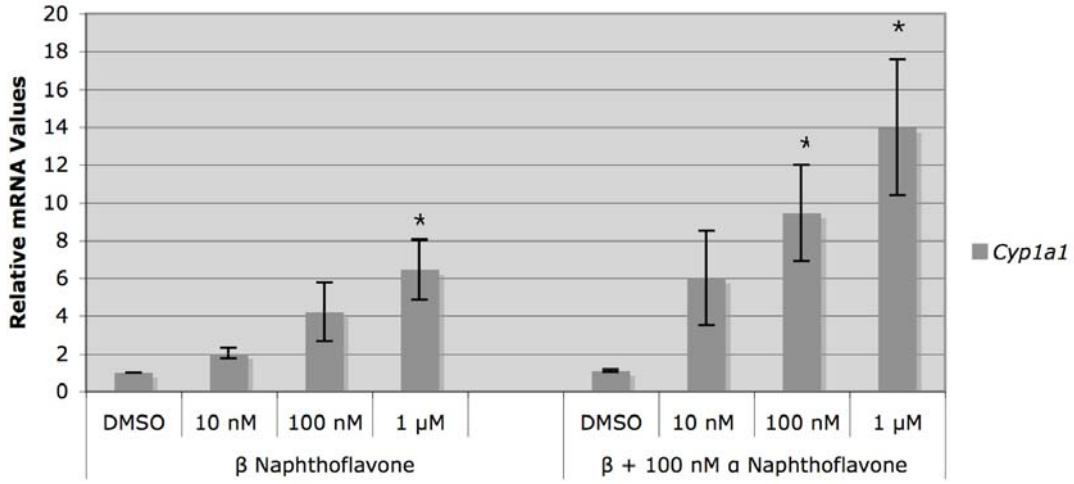
In many ways, the more surprising findings of this study were demonstrated by the lack of effects on proliferation following β-naphthoflavone treatment. There is

considerable evidence showing that AhR activation can promote proliferation. *Ahr*$^{+/+}$ mouse mammary fibroblasts are more tumorigenic than *Ahr*$^{-/-}$ cells (162), and AIP knockdown in GH3 cells, which presumably allows enhanced AhR transcription, promotes enhanced proliferation three days after transfection (150). Also, mutations in AIP have been identified as a cause of familial growth hormone secreting pituitary adenomas (51). Further relevant to the data presented here, there is an interesting inverse relationship with respect to AhR and TGFβ levels on proliferation. Experiments using primary hepatocytes found increased TGFβ secretions from *Ahr*$^{-/-}$ cells and also had lower proliferation rates than wild type (168). Additionally, adding a TGFβ neutralizing antibody to *Ahr*$^{-/-}$ fibroblasts restores proliferation close to wild type levels (162). It has been known for some time that TGFβ can suppress estrogen-induced lactrotrope proliferation (169), and these actions are modulated by dopaminergic hypothalamic input (170). It is tempting to speculate lactrotrope hyperplasia responsive to TGFβ administration, may also involve altered AhR or AIP activity. In fact, it is possible the proliferative effects of AIP silencing occurs in an AhR-independent manner as others have shown AIP interacts with phosphodiesterase4A5 and may affect proliferation by altering cAMP levels(49, 150) The results described here demonstrating a strong suppression of *TGFβ,* concomitant with a lack of proliferation changes following AhR activation are unexpected given the previous data presented above. However, it is possible more efficacious AhR ligands can induce cell cycle progression in GH3 cells, or much higher doses of β-naphthoflavone would be required than what was used in this study.

Many questions remain to be answered, however the present study demonstrates ligand-induced AhR can alter transcription of many pituitary hormones and has little effect on pituitary cell proliferation in culture. Future studies examining different AhR ligand effects on pituitary hormone response as well as further gene transcription changes in AhR$^{-/-}$ mouse pituitaries will be highly beneficial to the endocrine toxicology field.

Figure 4.1

A.


Relative mRNA Values
20
18
16
14
12
10
8
6
4
2
0
DMSO
10 nM
100 nM
1 µM
β Naphthoflavone
DMSO
10 nM
100 nM
1 µM
β + 100 nM α Naphthoflavone
Cyp1a1

B.

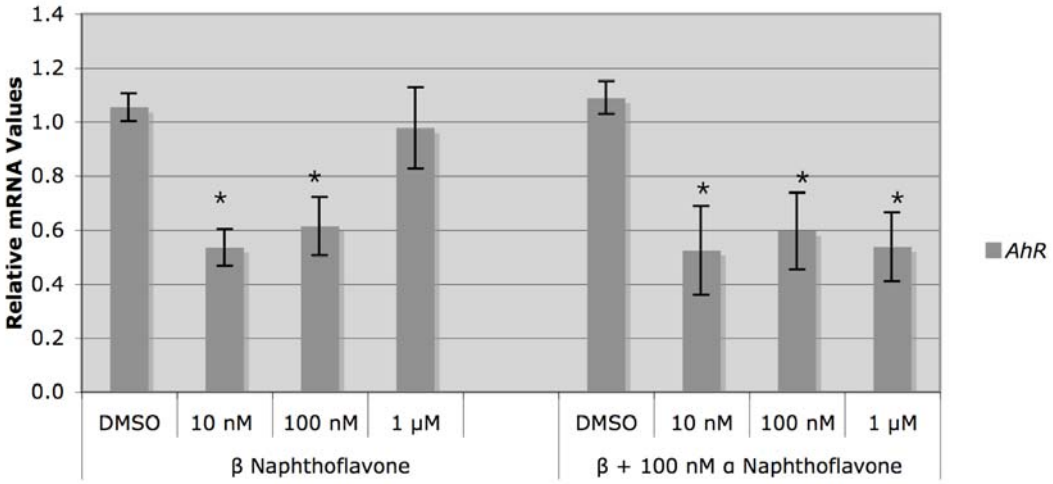
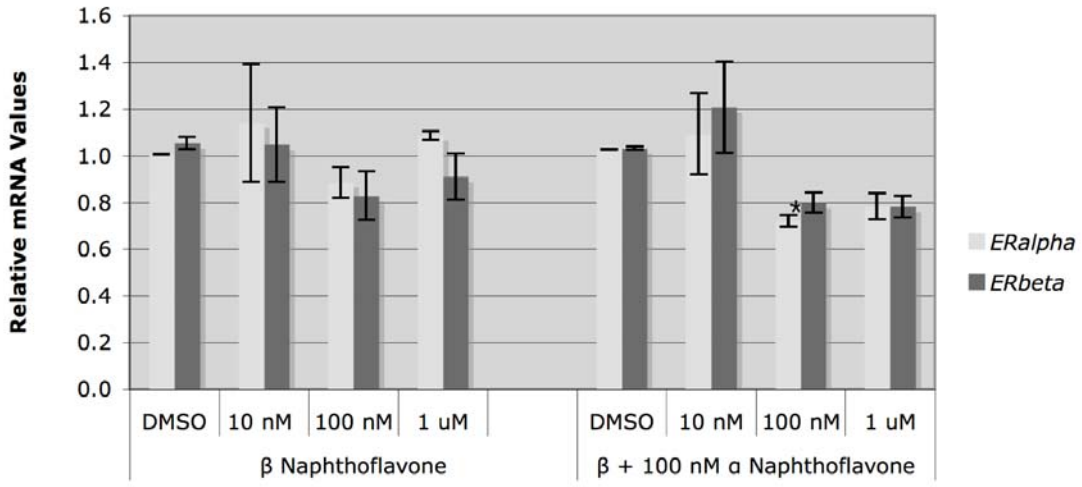

Relative mRNA Values
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
10 nM
100 nM
1 µM
β Naphthoflavone
DMSO
10 nM
100 nM
1 µM
β + 100 nM α Naphthoflavone
AhR

Figure 4.1 (cont.)

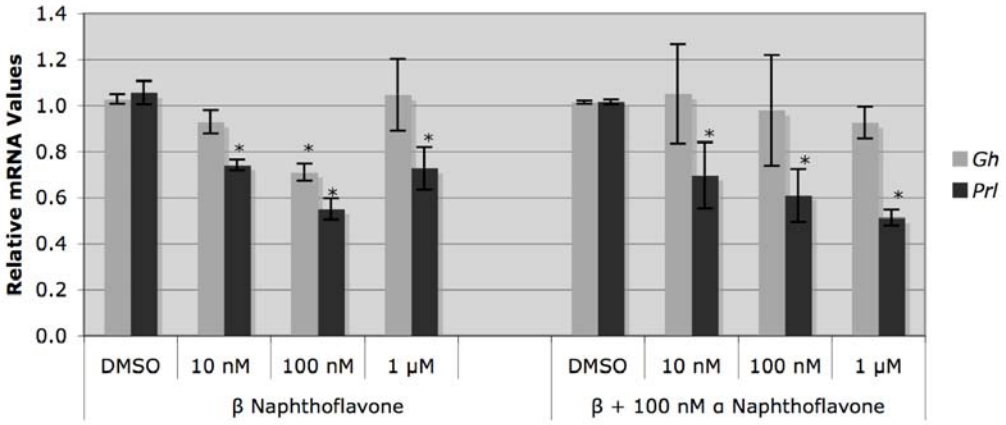
C.



β-naphthoflavone alone and with α-naphthoflavone activates *Cyp1a1*, suppresses *AhR* expression, and does not dramatically affect *Esr1*. Real time PCR shows significantly higher levels of *Cyp1a1* at 1 μM β-naphthoflavone and both 100 nM and 1 μM β-naphthoflavone plus 100 nM α-naphthoflavone (1a). *AhR* is significantly reduced at 10 nM and 100 nM β-naphthoflavone and at all doses of β-naphthoflavone plus α-naphthoflavone in cultured GH3 cells (1b), while β-naphthoflavone has only a small suppressive effect on *Esr1* (ERα) at 100 nM β-naphthoflavone plus 100 nM α-naphthoflavone (1c). mRNA values normalized to *beta-actin*, cells treated for 20 hours in charcoal dextran treated media, n=3.

Figure 4.2

A.


Relative mRNA Values
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
10 nM
100 nM
1 μM
β Naphthoflavone
DMSO
10 nM
100 nM
1 μM
β + 100 nM α Naphthoflavone
Gh
Prl

B.

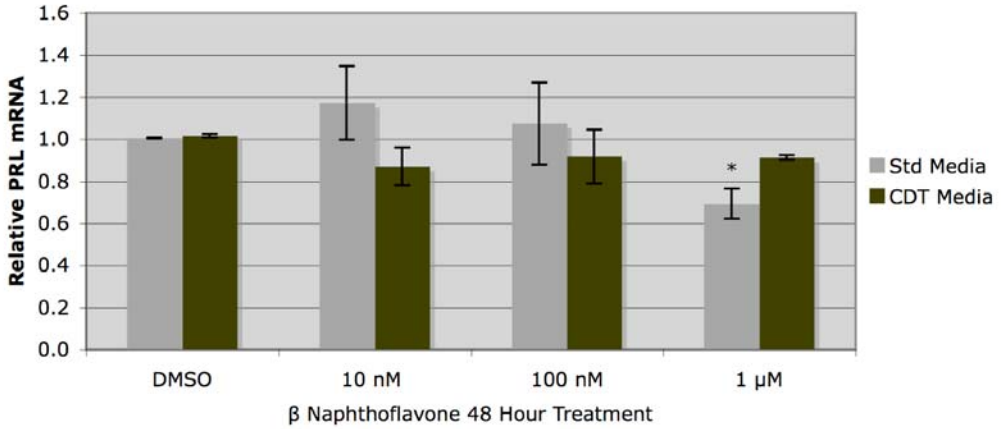
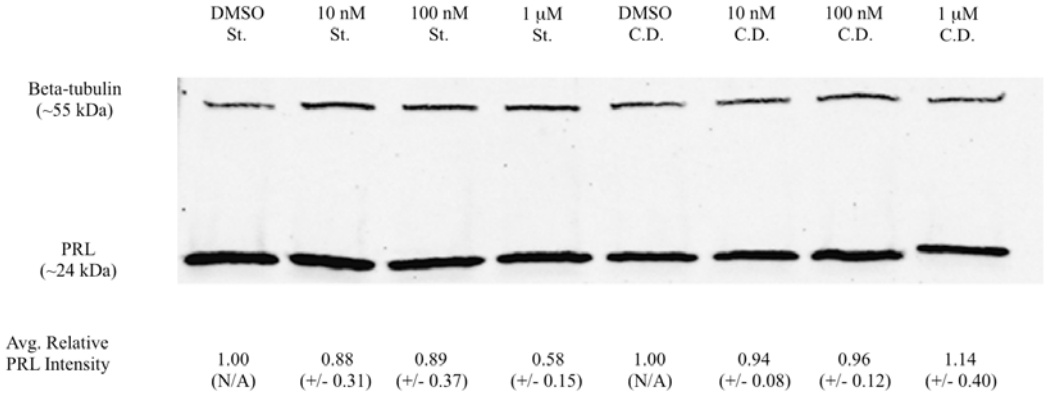

Relative PRL mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
DMSO
10 nM
100 nM
1 μM
β Naphthoflavone 48 Hour Treatment
Std Media
CDT Media

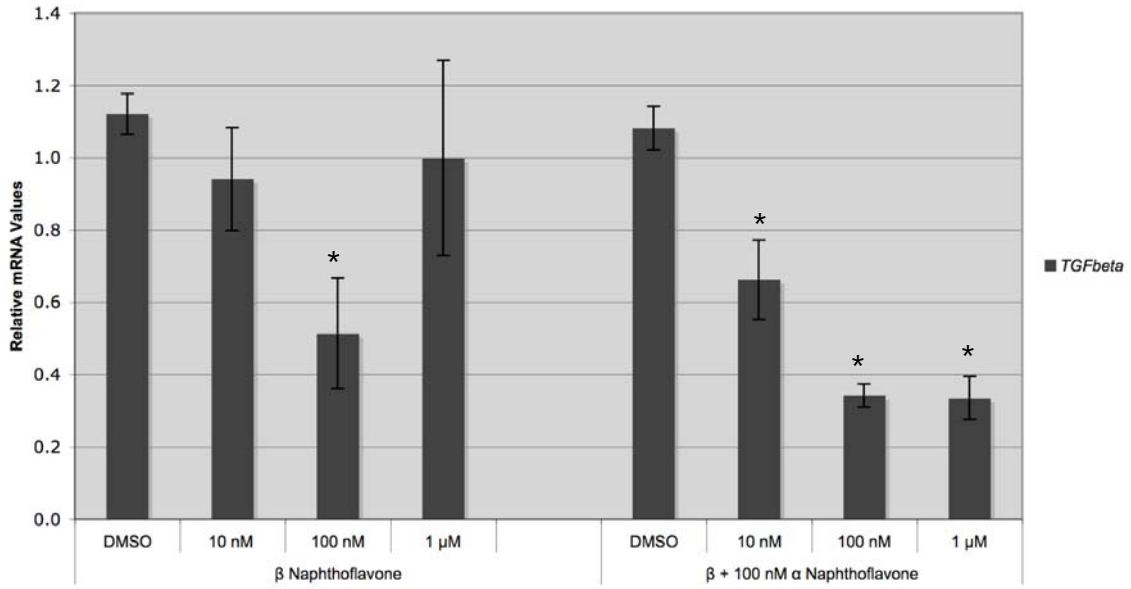
Figure 4.2 (cont.)

C.



Growth hormone and prolactin are suppressed via AhR. Growth hormone mRNA levels remained largely unchanged except at 100 nM β-naphthoflavone where they are significantly reduced. *Prl*, however, was significantly reduced with all doses of β-naphthoflavone and of β-naphthoflavone plus α-naphthoflavone in cultured GH3 cells (2a). mRNA values normalized to *beta-actin*, cells treated for 20 hours, n=3. Following 48 hours treatment in cultured GH3 cells, *Prl* is suppressed only at 1 μM β-naphthoflavone in standard growth media (2b). Western blot analysis following 48 hour treatment in cultured GH3 cells shows PRL is reduced in standard growth media (St.) but not in charcoal dextran treated media (C.D.) at 1 μM β-naphthoflavone (2c). Average relative band intensities compared to *β-tubulin* shown with standard error of mean, n=3.

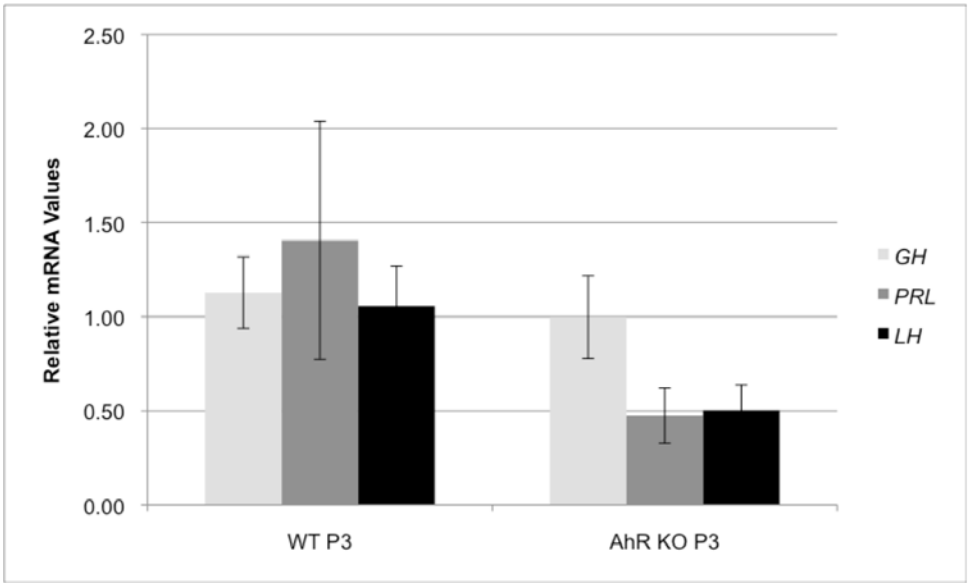
Figure 4.3



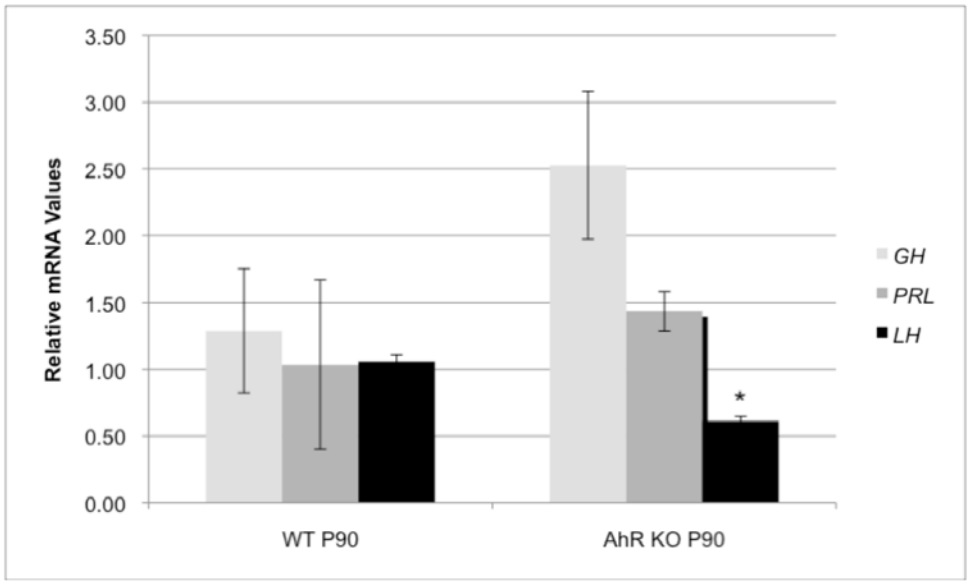
AhR suppresses TGFβ mRNA expression. Significant reduction in TGFβ mRNA occurs at 10 nM and 100 nM β-naphthoflavone and all doses of β-naphthoflavone plus α-naphthoflavone in cultured GH3 cells treated for 20 hours in charcoal dextran treated media. mRNA values normalized to *beta-actin*, n=3.

Figure 4.4

A.



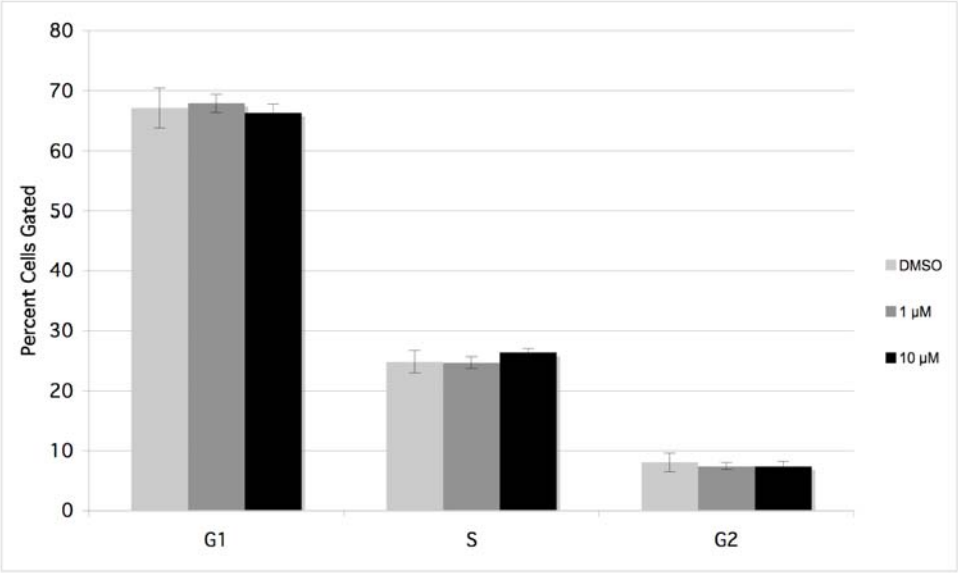
B.



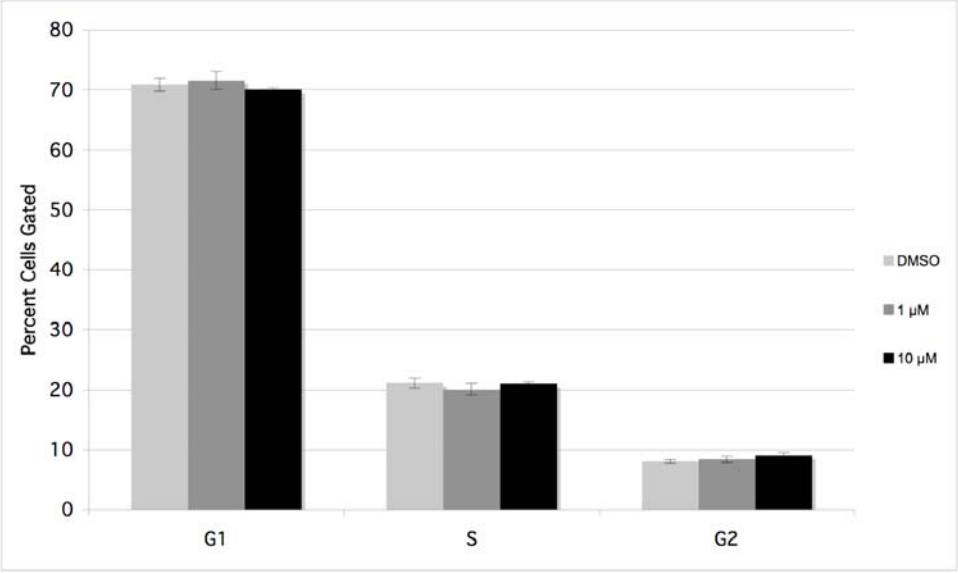
AhR$^{-/-}$ females have reduced LH expression at P90. Real time PCR performed on isolated P3 pituitaries from *Ahr*$^{-/-}$ and wildtype littermates shows a trend towards decreased *Prl* and *Lh* (4a), n=3. RTPCR analysis on isolated pituitaries from P90 females shows a trend towards increased *Gh* and significantly decreased *Lhb* ($p < 0.001$) (4b). mRNA values normalized to *GAPDH*, n=3.

Figure 4.5

A. Propidium Iodide Flow Analysis of Cells Grown in Standard Media



B. Propidium Iodide Flow Analysis of Cells Grown in Charcoal Dextran Treated Media



AhR activation via β-naphthoflavone does not affect proliferative capacity of GH3 pituitary cells. β-naphthoflavone at 1 and 10 μM does not alter the percentage of GH3 cells in G1, S, or G2 phases of the cell cycle in either standard (5a) or charcoal dextran treated serum medias (5b). Cultured GH3 cells with charcoal dextran treated serum had phenol red free media. Staining occurred by propidium iodide, and cell cycle analyzed by FACS, n=3.

Chapter Five: Concluding Remarks

The principal findings of Chapters Two and Three include the identification of a previously unknown role for the proteins Numb and Numblike during mouse pituitary development and adulthood. At the time this project began, Numb was mainly characterized as an inhibitor of Notch proteins in neuronal progenitors, influencing neuronal progenitor asymmetric cell division. It is now recognized to be an endocytic adaptor protein with a broad range of effects in many tissues, with certainly many more functions yet to be uncovered. The results here show that conditional loss of *Numb* and *Numblike* in POMC expressing cells results in pituitary intermediate lobe metaplasia, without evidence of hyperplasia. We therefore believe the primary role of Numb in the postnatal pituitary intermediate lobe is to maintain stability of adherens junctions proteins including N-cadherin, E-cadherin, β-catenin, and α-catenin as depicted in Figure 5.1. These results are not surprising, given the previously described role of Numb and adherens junctions proteins in the brain.

Three major aspects of this study presented in Chapter Two, which were unexpected and certainly warrant future investigation, are the lack of observed differences in proliferation, cell fate commitment, and Notch activity in P30 cDKO compared to control pituitaries. As shown in previous studies, Numb appears to alter proliferation through one of two major mechanisms. The first, and representing significant recent interest, is from observations of cancer tissues from human patients with low or absent levels of Numb compared to normal tissue. This was also shown to be due to loss of p53, as Numb can protect p53 ubiquitination by binding and inactivating the ubiquitin ligase MDM2. We did not address p53 in this study, however, further experiments utilizing co-immunoprecipitations in primary tissues to find if NUMB is directly associating with p53 in the pituitary would be useful. It is known that p53 deficient mice do not get pituitary tumors, but loss of one or two copies can accelerate tumor progression in mice heterozygous for the *Retinoblastoma* gene (*Rb*+/-). This result suggests these cDKO animals may be more susceptible to developing tumors. It is interesting to speculate that like tumors of other origins, pituitary adenomas may arise from loss of Numb, destabilization of p53, concomitant with another unknown cell cycle protein alteration. The second known major mechanism by which Numb can alter

proliferation is due to changes in progenitor cell populations, following asymmetric division from studies utilizing early conditional *Numb* deletion in neuronal precursors. The isoform data we present, show the longer forms of the proline rich region (PRR) which is associated with progenitor cells in other contexts, is most highly expressed in embryonic pituitaries whereas the shorter PRR isoform is more highly expressed in adulthood. It would be extremely interesting to utilize a pituitary progenitor cell specific Cre-recombinase transgenic mouse to conditionally delete Numb in pituitary progenitor cells before hormone cell differentiation occurs, and track progenitor cell proliferation, as well as hormone cell differentiation with immunohistochemistry. Last, alterations in Notch activity determined by immunohistochemistry, *in situ* hybridization, or laser capture microdissection and RTPCR, could determine if Numb does alter Notch activity early in pituitary development, and if these changes coincide with the hormone cell differentiation changes. Last, these experiments could be supplemented by cell culture experiments using either the somatolactotrope, gonadotrope, or corticotrope cell lines, whereby Numb expression is knocked down via siRNA or lenti-viral vector, specific isoforms could be re-expressed, and changes in Notch downstream targets could be quantified by RTPCR concomitant with mRNA changes in pituitary hormones, cell adhesion, or other markers. These experiments may yield great insight into the actions of transient Notch activity, which immediately precede pituitary hormone cell differentiation.

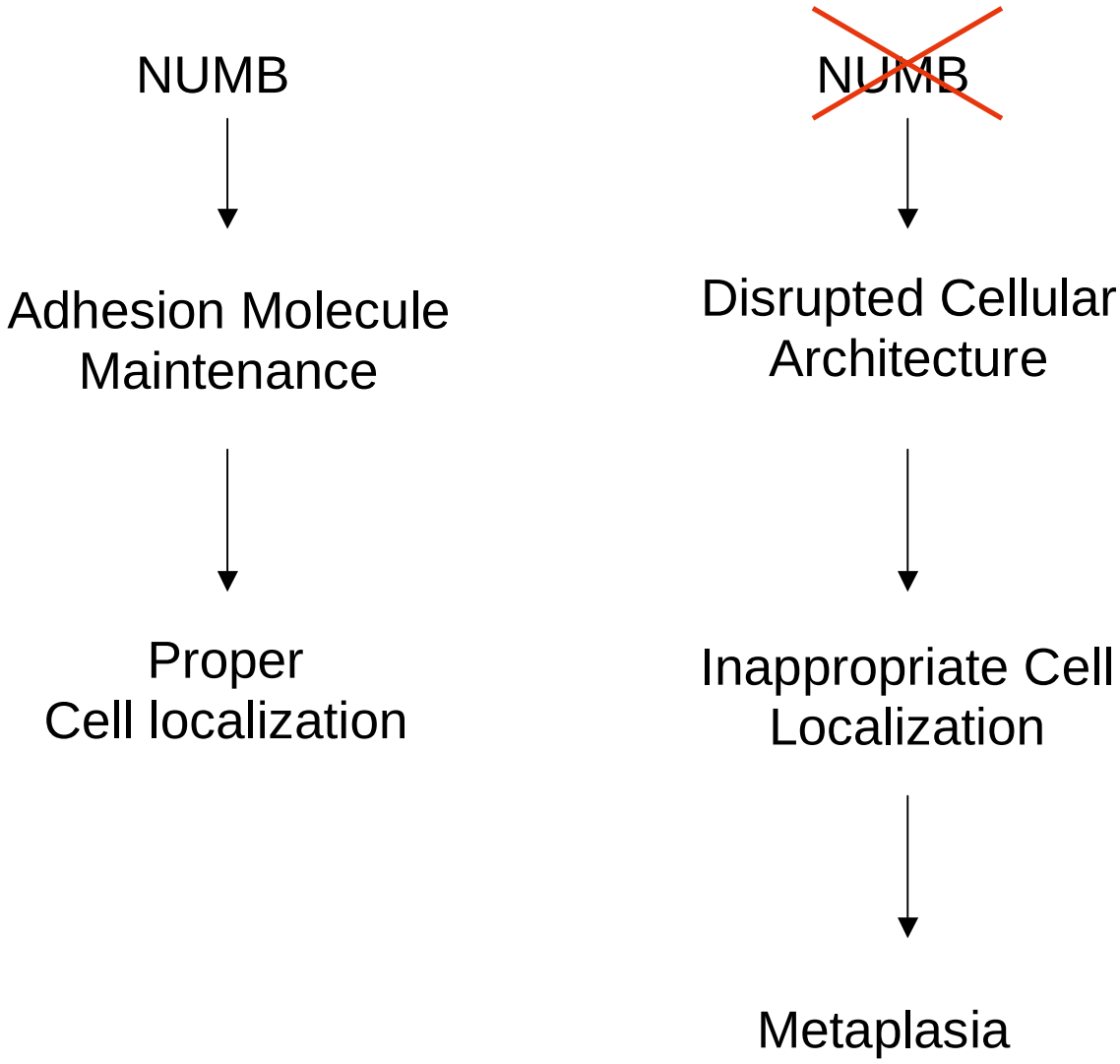
Preliminary findings related to an additional role of Numb expression in the mammalian pituitary are described in Chapter Three, and related to a putative role in gonadotrope function. We identify Numb as a protein which is enriched in most, but not all LH and FSH positive cells of the mouse pituitary. Further, we have preliminary evidence that LH mRNA is suppressed in male cDKO pituitaries using the αGSU-Cre transgenic. Current ongoing studies include identifying LH mRNA changes in female mice as well as female cDKO date of vaginal openings, frequency of estrous cycles, litter size, time period between pregnancies, and pup survivals. Finally, both male and female cDKO and controls will be sacrificed for reproductive organ pathology, including testes, ovaries and pituitaries. These studies have potential to be a highly unique contribution to

the reproductive field, and represent the first description of a reproductive phenotype to the protein Numb.

The final research chapter (Four) presents an interesting role for the aryl hydrocarbon receptor both as a mediator of exogenous endocrine disruptors, but also as a necessary endogenous component of pituitary physiology. Interestingly, we find that not only did the putative AhR antagonist α-naphthoflavone have synergistic effects at 100 nM with agonist β-naphthoflavone in the pituitary GH3 somatolactotrope cell line, but these ligands suppressed prolactin, and not growth hormone. These results are important, as they show nanomolar doses of relatively low affinity AhR ligands are capable of acting as endocrine disruptors. Additional studies to determine the mechanism by which this occurs could include chromatin-immunoprecipitation and identify if AhR is bound to xenobiotic response elements proximal to the PRL and GH promoter sequences.

AhR has also been shown to have a subtle, yet intriguing role in normal pituitary function. The real-time PCR data from the $AhR^{-/-}$ pituitaries shows that at P3, LH levels are exhibiting a trend towards being low, and by P90 are significantly reduced. These results support and may play a role in the impaired ovarian follicular development in these mice as described by Benedict et al. (2000). At P90, the $AhR^{-/-}$ pituitary cells are fully differentiated, and would more closely be correlated to the β-naphthoflavone cell culture experiments. Interestingly, PRL and especially GH show trends towards increased expression in P90 $AhR^{-/-}$ pituitaries. This might be expected for PRL which was shown to be decreased with AhR activation, however is contrary to the lack of effect on GH expression we found in the culture experiments. Future studies should include analyzing more $AhR^{-/-}$ pituitaries, increasing sample size at both P3 and P90, but also looking at post pubertal female, as well as male, ages. Additionally, a basic pathology analysis of these pituitaries could yield interesting data concerning onset, as well as localization of hormone producing cells. Finally, despite the lack of evidence showing proliferative changes in culture following AhR activation though β-naphthoflavone, proliferation of one or more particular hormone producing cell types may be altered and should be analyzed, along with co-immunostaining for AIP and ARNT.

Figure 5.1



Schematic of Numb actions in the pituitary intermediate lobe.

References

1. Uemura T, Shepherd S, Ackerman L, Jan LY, Jan YN 1989 numb, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. Cell 58:349-360
2. Zhong W, Feder JN, Jiang MM, Jan LY, Jan YN 1996 Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. Neuron 17:43-53
3. Bani-Yaghoub M, Kubu CJ, Cowling R, Rochira J, Nikopoulos GN, Bellum S, Verdi JM 2007 A switch in numb isoforms is a critical step in cortical development. Dev Dyn 236:696-705
4. Corallini S, Fera S, Grisanti L, Falciatori I, Muciaccia B, Stefanini M, Vicini E 2006 Expression of the adaptor protein m-Numb in mouse male germ cells. Reproduction 132:887-897
5. Yoshida T, Tokunaga A, Nakao K, Okano H 2003 Distinct expression patterns of splicing isoforms of mNumb in the endocrine lineage of developing pancreas. Differentiation 71:486-495
6. Knoblich JA, Jan LY, Jan YN 1997 The N terminus of the Drosophila Numb protein directs membrane association and actin-dependent asymmetric localization. Proc Natl Acad Sci U S A 94:13005-13010
7. Jan YN, Jan LY 1998 Asymmetric cell division. Nature 392:775-778
8. Dho SE, French MB, Woods SA, McGlade CJ 1999 Characterization of four mammalian numb protein isoforms. Identification of cytoplasmic and membrane-associated variants of the phosphotyrosine binding domain. J Biol Chem 274:33097-33104
9. Dho SE, Trejo J, Siderovski DP, McGlade CJ 2006 Dynamic regulation of mammalian numb by G protein-coupled receptors and protein kinase C activation: Structural determinants of numb association with the cortical membrane. Mol Biol Cell 17:4142-4155
10. Salcini AE, Confalonieri S, Doria M, Santolini E, Tassi E, Minenkova O, Cesareni G, Pelicci PG, Di Fiore PP 1997 Binding specificity and in vivo targets of the EH domain, a novel protein-protein interaction module. Genes Dev 11:2239-2249
11. Santolini E, Puri C, Salcini AE, Gagliani MC, Pelicci PG, Tacchetti C, Di Fiore PP 2000 Numb is an endocytic protein. J Cell Biol 151:1345-1352
12. Smith CA, Dho SE, Donaldson J, Tepass U, McGlade CJ 2004 The cell fate determinant numb interacts with EHD/Rme-1 family proteins and has a role in endocytic recycling. Mol Biol Cell 15:3698-3708
13. McGill MA, Dho SE, Weinmaster G, McGlade CJ 2009 Numb regulates post-endocytic trafficking and degradation of Notch1. J Biol Chem 284:26427-26438
14. Zhong W, Jiang MM, Weinmaster G, Jan LY, Jan YN 1997 Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. Development 124:1887-1897
15. Spana EP, Kopczynski C, Goodman CS, Doe CQ 1995 Asymmetric localization of numb autonomously determines sibling neuron identity in the Drosophila CNS. Development 121:3489-3494

16. Rhyu MS, Jan LY, Jan YN 1994 Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell 76:477-491
17. Zhong W, Jiang MM, Schonemann MD, Meneses JJ, Pedersen RA, Jan LY, Jan YN 2000 Mouse numb is an essential gene involved in cortical neurogenesis. Proc Natl Acad Sci U S A 97:6844-6849
18. Zilian O, Saner C, Hagedorn L, Lee HY, Sauberli E, Suter U, Sommer L, Aguet M 2001 Multiple roles of mouse Numb in tuning developmental cell fates. Curr Biol 11:494-501
19. Petersen PH, Zou K, Hwang JK, Jan YN, Zhong W 2002 Progenitor cell maintenance requires numb and numblike during mouse neurogenesis. Nature 419:929-934
20. Li HS, Wang D, Shen Q, Schonemann MD, Gorski JA, Jones KR, Temple S, Jan LY, Jan YN 2003 Inactivation of Numb and Numblike in embryonic dorsal forebrain impairs neurogenesis and disrupts cortical morphogenesis. Neuron 40:1105-1118
21. Petersen PH, Zou K, Krauss S, Zhong W 2004 Continuing role for mouse Numb and Numbl in maintaining progenitor cells during cortical neurogenesis. Nat Neurosci 7:803-811
22. Shen Q, Zhong W, Jan YN, Temple S 2002 Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. Development 129:4843-4853
23. Verdi JM, Bashirullah A, Goldhawk DE, Kubu CJ, Jamali M, Meakin SO, Lipshitz HD 1999 Distinct human NUMB isoforms regulate differentiation vs. proliferation in the neuronal lineage. Proc Natl Acad Sci U S A 96:10472-10476
24. Li SC, Zwahlen C, Vincent SJ, McGlade CJ, Kay LE, Pawson T, Forman-Kay JD 1998 Structure of a Numb PTB domain-peptide complex suggests a basis for diverse binding specificity. Nat Struct Biol 5:1075-1083
25. Siegal G 1999 The surprisingly flexible PTB domain. Nat Struct Biol 6:7-10
26. Wakamatsu Y, Maynard TM, Jones SU, Weston JA 1999 NUMB localizes in the basal cortex of mitotic avian neuroepithelial cells and modulates neuronal differentiation by binding to NOTCH-1. Neuron 23:71-81
27. Artavanis-Tsakonas S, Rand MD, Lake RJ 1999 Notch signaling: cell fate control and signal integration in development. Science 284:770-776
28. Jarriault S, Brou C, Logeat F, Schroeter EH, Kopan R, Israel A 1995 Signalling downstream of activated mammalian Notch. Nature 377:355-358
29. Kageyama R, Nakanishi S 1997 Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. Curr Opin Genet Dev 7:659-665
30. McGill MA, McGlade CJ 2003 Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. J Biol Chem 278:23196-23203
31. Chastagner P, Israel A, Brou C 2008 AIP4/Itch regulates Notch receptor degradation in the absence of ligand. PLoS One 3:e2735
32. Qiu L, Joazeiro C, Fang N, Wang HY, Elly C, Altman Y, Fang D, Hunter T, Liu YC 2000 Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. J Biol Chem 275:35734-35737

33. Rasin MR, Gazula VR, Breunig JJ, Kwan KY, Johnson MB, Liu-Chen S, Li HS, Jan LY, Jan YN, Rakic P, Sestan N 2007 Numb and Numbl are required for maintenance of cadherin-based adhesion and polarity of neural progenitors. Nat Neurosci 10:819-827
34. Nishimura T, Kaibuchi K 2007 Numb controls integrin endocytosis for directional cell migration with aPKC and PAR-3. Dev Cell 13:15-28
35. Zhou Y, Atkins JB, Rompani SB, Bancescu DL, Petersen PH, Tang H, Zou K, Stewart SB, Zhong W 2007 The mammalian Golgi regulates numb signaling in asymmetric cell division by releasing ACBD3 during mitosis. Cell 129:163-178
36. Colaluca IN, Tosoni D, Nuciforo P, Senic-Matuglia F, Galimberti V, Viale G, Pece S, Di Fiore PP 2008 NUMB controls p53 tumour suppressor activity. Nature 451:76-80
37. Pece S, Serresi M, Santolini E, Capra M, Hulleman E, Galimberti V, Zurrida S, Maisonneuve P, Viale G, Di Fiore PP 2004 Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. J Cell Biol 167:215-221
38. Hahn H, Wojnowski L, Miller G, Zimmer A 1999 The patched signaling pathway in tumorigenesis and development: lessons from animal models. J Mol Med 77:459-468
39. Yoon JW, Kita Y, Frank DJ, Majewski RR, Konicek BA, Nobrega MA, Jacob H, Walterhouse D, Iannaccone P 2002 Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. J Biol Chem 277:5548-5555
40. Di Marcotullio L, Ferretti E, Greco A, De Smaele E, Po A, Sico MA, Alimandi M, Giannini G, Maroder M, Screpanti I, Gulino A 2006 Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. Nat Cell Biol 8:1415-1423
41. Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, Pelosi G, Spaggiari L, Mazzarol G, Viale G, Pece S, Di Fiore PP 2009 Alterations of the Notch pathway in lung cancer. Proc Natl Acad Sci U S A 106(52):22293-8
42. Raetzman LT, Ross SA, Cook S, Dunwoodie SL, Camper SA, Thomas PQ 2004 Developmental regulation of Notch signaling genes in the embryonic pituitary: Prop1 deficiency affects Notch2 expression. Dev Biol 265:329-340
43. Raetzman LT, Wheeler BS, Ross SA, Thomas PQ, Camper SA 2006 Persistent expression of Notch2 delays gonadotrope differentiation. Mol Endocrinol 20:2898-2908
44. Chauvet N, El-Yandouzi T, Mathieu MN, Schlernitzauer A, Galibert E, Lafont C, Le Tissier P, Robinson IC, Mollard P, Coutry N 2009 Characterization of adherens junction protein expression and localization in pituitary cell networks. J Endocrinol 202:375-387
45. Rubinek T, Yu R, Hadani M, Barkai G, Nass D, Melmed S, Shimon I 2003 The cell adhesion molecules N-cadherin and neural cell adhesion molecule regulate human growth hormone: a novel mechanism for regulating pituitary hormone secretion. J Clin Endocrinol Metab 88:3724-3730
46. Chen J, Gremeaux L, Fu Q, Liekens D, Van Laere S, Vankelecom H 2009 Pituitary progenitor cells tracked down by side population dissection. Stem Cells 27:1182-1195
47. Alam MS, Maekawa Y, Kitamura A, Tanigaki K, Yoshimoto T, Kishihara K, Yasutomo K 2010 Notch signaling drives IL-22 secretion in CD4+ T cells by stimulating the aryl hydrocarbon receptor. Proc Natl Acad Sci U S A 107:5943-5948

48. Thomsen JS, Kietz S, Strom A, Gustafsson JA 2004 HES-1, a novel target gene for the aryl hydrocarbon receptor. Mol Pharmacol 65:165-171
49. Leontiou CA, Gueorguiev M, van der Spuy J, Quinton R, Lolli F, Hassan S, Chahal HS, Igreja SC, Jordan S, Rowe J, Stolbrink M, Christian HC, Wray J, Bishop-Bailey D, Berney DM, Wass JA, Popovic V, Ribeiro-Oliveira A,Jr, Gadelha MR, Monson JP, Akker SA, Davis JR, Clayton RN, Yoshimoto K, Iwata T, Matsuno A, Eguchi K, Musat M, Flanagan D, Peters G, Bolger GB, Chapple JP, Frohman LA, Grossman AB, Korbonits M 2008 The role of the aryl hydrocarbon receptor-interacting protein gene in familial and sporadic pituitary adenomas. J Clin Endocrinol Metab 93:2390-2401
50. Jaffrain-Rea ML, Angelini M, Gargano D, Tichomirowa MA, Daly AF, Vanbellinghen JF, D'Innocenzo E, Barlier A, Giangaspero F, Esposito V, Ventura L, Arcella A, Theodoropoulou M, Naves LA, Fajardo C, Zacharieva S, Rohmer V, Brue T, Gulino A, Cantore G, Alesse E, Beckers A 2009 Expression of aryl hydrocarbon receptor (AHR) and AHR-interacting protein in pituitary adenomas: pathological and clinical implications. Endocr Relat Cancer 16:1029-1043
51. Vierimaa O, Georgitsi M, Lehtonen R, Vahteristo P, Kokko A, Raitila A, Tuppurainen K, Ebeling TM, Salmela PI, Paschke R, Gundogdu S, De Menis E, Makinen MJ, Launonen V, Karhu A, Aaltonen LA 2006 Pituitary adenoma predisposition caused by germline mutations in the AIP gene. Science 312:1228-1230
52. Gao X, Son DS, Terranova PF, Rozman KK 1999 Toxic equivalency factors of polychlorinated dibenzo-p-dioxins in an ovulation model: validation of the toxic equivalency concept for one aspect of endocrine disruption. Toxicol Appl Pharmacol 157:107-116
53. Huang P, Ceccatelli S, Hakansson H, Grandison L, Rannug A 2002 Constitutive and TCDD-induced expression of Ah receptor-responsive genes in the pituitary. Neurotoxicology 23:783-793
54. Elango A, Shepherd B, Chen TT 2006 Effects of endocrine disrupters on the expression of growth hormone and prolactin mRNA in the rainbow trout pituitary. Gen Comp Endocrinol 145:116-127
55. Aluru N, Vijayan MM 2008 Brain transcriptomics in response to beta-naphthoflavone treatment in rainbow trout: the role of aryl hydrocarbon receptor signaling. Aquat Toxicol 87:1-12
56. Vankelecom H, Gremeaux L 2010 Stem cells in the pituitary gland: A burgeoning field. Gen Comp Endocrinol 166:478-488
57. Japon MA, Rubinstein M, Low MJ 1994 In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J Histochem Cytochem 42:1117-1125
58. Kawamura K, Kouki T, Kawahara G, Kikuyama S 2002 Hypophyseal development in vertebrates from amphibians to mammals. Gen Comp Endocrinol 126:130-135
59. Thor S, Ericson J, Brannstrom T, Edlund T 1991 The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat. Neuron 7:881-889

60. Roberson MS, Schoderbek WE, Tremml G, Maurer RA 1994 Activation of the glycoprotein hormone alpha-subunit promoter by a LIM-homeodomain transcription factor. Mol Cell Biol 14:2985-2993
61. Sheng HZ, Moriyama K, Yamashita T, Li H, Potter SS, Mahon KA, Westphal H 1997 Multistep control of pituitary organogenesis. Science 278:1809-1812
62. Ericson J, Norlin S, Jessell TM, Edlund T 1998 Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. Development 125:1005-1015
63. De Moerlooze L, Spencer-Dene B, Revest JM, Hajihosseini M, Rosewell I, Dickson C 2000 An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. Development 127:483-492
64. Ohuchi H, Hori Y, Yamasaki M, Harada H, Sekine K, Kato S, Itoh N 2000 FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. Biochem Biophys Res Commun 277:643-649
65. Treier M, Gleiberman AS, O'Connell SM, Szeto DP, McMahon JA, McMahon AP, Rosenfeld MG 1998 Multistep signaling requirements for pituitary organogenesis in vivo. Genes Dev 12:1691-1704
66. Treier M, O'Connell S, Gleiberman A, Price J, Szeto DP, Burgess R, Chuang PT, McMahon AP, Rosenfeld MG 2001 Hedgehog signaling is required for pituitary gland development. Development 128:377-386
67. Zhu X, Zhang J, Tollkuhn J, Ohsawa R, Bresnick EH, Guillemot F, Kageyama R, Rosenfeld MG 2006 Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis. Genes Dev 20:2739-2753
68. Raetzman LT, Cai JX, Camper SA 2007 Hes1 is required for pituitary growth and melanotrope specification. Dev Biol 304:455-466
69. Li S, Crenshaw EB, 3rd, Rawson EJ, Simmons DM, Swanson LW, Rosenfeld MG 1990 Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. Nature 347:528-533
70. Camper SA, Saunders TL, Katz RW, Reeves RH 1990 The Pit-1 transcription factor gene is a candidate for the murine Snell dwarf mutation. Genomics 8:586-590
71. Nasonkin IO, Ward RD, Raetzman LT, Seasholtz AF, Saunders TL, Gillespie PJ, Camper SA 2004 Pituitary hypoplasia and respiratory distress syndrome in Prop1 knockout mice. Hum Mol Genet 13:2727-2735
72. Himes AD, Raetzman LT 2009 Premature differentiation and aberrant movement of pituitary cells lacking both Hes1 and Prop1. Dev Biol 325:151-161
73. Chen J, Hersmus N, Van Duppen V, Caesens P, Denef C, Vankelecom H 2005 The adult pituitary contains a cell population displaying stem/progenitor cell and early embryonic characteristics. Endocrinology 146:3985-3998
74. Chen J, Crabbe A, Van Duppen V, Vankelecom H 2006 The notch signaling system is present in the postnatal pituitary: marked expression and regulatory activity in the newly discovered side population. Mol Endocrinol 20:3293-3307
75. Gleiberman AS, Michurina T, Encinas JM, Roig JL, Krasnov P, Balordi F, Fishell G, Rosenfeld MG, Enikolopov G 2008 Genetic approaches identify adult pituitary stem cells. Proc Natl Acad Sci U S A 105:6332-6337

76. Fauquier T, Rizzoti K, Dattani M, Lovell-Badge R, Robinson IC 2008 SOX2-expressing progenitor cells generate all of the major cell types in the adult mouse pituitary gland. Proc Natl Acad Sci U S A 105:2907-2912
77. Evans CO, Moreno CS, Zhan X, McCabe MT, Vertino PM, Desiderio DM, Oyesiku NM 2008 Molecular pathogenesis of human prolactinomas identified by gene expression profiling, RT-qPCR, and proteomic analyses. Pituitary 11:231-245
78. Moreno CS, Evans CO, Zhan X, Okor M, Desiderio DM, Oyesiku NM 2005 Novel molecular signaling and classification of human clinically nonfunctional pituitary adenomas identified by gene expression profiling and proteomic analyses. Cancer Res 65:10214-10222
79. Wodarz A, Ramrath A, Kuchinke U, Knust E 1999 Bazooka provides an apical cue for Inscuteable localization in Drosophila neuroblasts. Nature 402:544-547
80. Wang Z, Sandiford S, Wu C, Li SS 2009 Numb regulates cell-cell adhesion and polarity in response to tyrosine kinase signalling. EMBO J 28:2360-2373
81. Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, Pelosi G, Spaggiari L, Mazzarol G, Viale G, Pece S, Di Fiore PP 2009 Alterations of the Notch pathway in lung cancer. Proc Natl Acad Sci U S A 106(52):22293-8
82. Maiorano E, Favia G, Pece S, Resta L, Maisonneuve P, Di Fiore PP, Capodiferro S, Urbani U, Viale G 2007 Prognostic implications of NUMB immunoreactivity in salivary gland carcinomas. Int J Immunopathol Pharmacol 20:779-789
83. Balthasar N, Coppari R, McMinn J, Liu SM, Lee CE, Tang V, Kenny CD, McGovern RA, Chua SC,Jr, Elmquist JK, Lowell BB 2004 Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron 42:983-991
84. Goudreau JL, Falls WM, Lookingland KJ, Moore KE 1995 Periventricular-hypophysial dopaminergic neurons innervate the intermediate but not the neural lobe of the rat pituitary gland. Neuroendocrinology 62:147-154
85. Kawano H, Daikoku S 1987 Functional topography of the rat hypothalamic dopamine neuron systems: retrograde tracing and immunohistochemical study. J Comp Neurol 265:242-253
86. Leranth C, Palkovits M, Krieger DT 1983 Serotonin immunoreactive nerve fibers and terminals in the rat pituitary--light- and electron-microscopic studies. Neuroscience 9:289-296
87. Mezey E, Leranth C, Brownstein MJ, Friedman E, Krieger DT, Palkovits M 1984 On the origin of the serotonergic input to the intermediate lobe of the rat pituitary. Brain Res 294:231-237
88. Oertel WH, Mugnaini E, Tappaz ML, Weise VK, Dahl AL, Schmechel DE, Kopin IJ 1982 Central GABAergic innervation of neurointermediate pituitary lobe: biochemical and immunocytochemical study in the rat. Proc Natl Acad Sci U S A 79:675-679
89. McNay DE, Pelling M, Claxton S, Guillemot F, Ang SL 2006 Mash1 is required for generic and subtype differentiation of hypothalamic neuroendocrine cells. Mol Endocrinol 20:1623-1632
90. Parton LE, Ye CP, Coppari R, Enriori PJ, Choi B, Zhang CY, Xu C, Vianna CR, Balthasar N, Lee CE, Elmquist JK, Cowley MA, Lowell BB 2007 Glucose sensing by POMC neurons regulates glucose homeostasis and is impaired in obesity. Nature 449:228-232

91. Korteweg N, Maia AS, Verhage M, Burbach JP 2004 Development of the mouse hypothalamo-neurohypophysial system in the munc18-1 null mutant that lacks regulated secretion. Eur J Neurosci 19:2944-2952
92. Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY, Nakayama K 1996 Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell 85:707-720
93. Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai LH, Broudy V, Perlmutter RM, Kaushansky K, Roberts JM 1996 A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell 85:733-744
94. Kiyokawa H, Kineman RD, Manova-Todorova KO, Soares VC, Hoffman ES, Ono M, Khanam D, Hayday AC, Frohman LA, Koff A 1996 Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell 85:721-732
95. Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A 1992 Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 359:288-294
96. Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA 1992 Effects of an Rb mutation in the mouse. Nature 359:295-300
97. Hu N, Gutsmann A, Herbert DC, Bradley A, Lee WH, Lee EY 1994 Heterozygous Rb-1 delta 20/+mice are predisposed to tumors of the pituitary gland with a nearly complete penetrance. Oncogene 9:1021-1027
98. Nikitin AY, Lee WH 1996 Early loss of the retinoblastoma gene is associated with impaired growth inhibitory innervation during melanotroph carcinogenesis in Rb+/- mice. Genes Dev 10:1870-1879
99. Stylianou S, Clarke RB, Brennan K 2006 Aberrant activation of notch signaling in human breast cancer. Cancer Res 66:1517-1525
100. Hollstein M, Sidransky D, Vogelstein B, Harris CC 1991 P53 Mutations in Human Cancers. Science 253:49-53
101. Harvey M, Vogel H, Lee EY, Bradley A, Donehower LA 1995 Mice deficient in both p53 and Rb develop tumors primarily of endocrine origin. Cancer Res 55:1146-1151
102. Wilson A, Ardiet DL, Saner C, Vilain N, Beermann F, Aguet M, Macdonald HR, Zilian O 2007 Normal hemopoiesis and lymphopoiesis in the combined absence of numb and numblike. J Immunol 178:6746-6751
103. Burrows HL, Douglas KR, Seasholtz AF, Camper SA 1999 Genealogy of the Anterior Pituitary Gland: Tracing a Family Tree. Trends Endocrinol Metab 10:343-352
104. Suh H, Gage PJ, Drouin J, Camper SA 2002 Pitx2 is required at multiple stages of pituitary organogenesis: pituitary primordium formation and cell specification. Development 129:329-337
105. Dasen JS, O'Connell SM, Flynn SE, Treier M, Gleiberman AS, Szeto DP, Hooshmand F, Aggarwal AK, Rosenfeld MG 1999 Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. Cell 97:587-598
106. Charles MA, Saunders TL, Wood WM, Owens K, Parlow AF, Camper SA, Ridgway EC, Gordon DF 2006 Pituitary-specific Gata2 knockout: effects on gonadotrope and thyrotrope function. Mol Endocrinol 20:1366-1377

107. Zhao L, Bakke M, Krimkevich Y, Cushman LJ, Parlow AF, Camper SA, Parker KL 2001 Steroidogenic factor 1 (SF1) is essential for pituitary gonadotrope function. Development 128:147-154
108. Topilko P, Schneider-Maunoury S, Levi G, Trembleau A, Gourdji D, Driancourt MA, Rao CV, Charnay P 1998 Multiple pituitary and ovarian defects in Krox-24 (NGFI-A, Egr-1)-targeted mice. Mol Endocrinol 12:107-122
109. Pulichino AM, Vallette-Kasic S, Tsai JP, Couture C, Gauthier Y, Drouin J 2003 Tpit determines alternate fates during pituitary cell differentiation. Genes Dev 17:738-747
110. Cushman LJ, Watkins-Chow DE, Brinkmeier ML, Raetzman LT, Radak AL, Lloyd RV, Camper SA 2001 Persistent Prop1 expression delays gonadotrope differentiation and enhances pituitary tumor susceptibility. Hum Mol Genet 10:1141-1153
111. Vesper AH, Raetzman LT, Camper SA 2006 Role of prophet of Pit1 (PROP1) in gonadotrope differentiation and puberty. Endocrinology 147:1654-1663
112. Cushman LJ, Burrows HL, Seasholtz AF, Lewandoski M, Muzyczka N, Camper SA 2000 Cre-mediated recombination in the pituitary gland. Genesis 28:167-174
113. Gieske MC, Kim HJ, Legan SJ, Koo Y, Krust A, Chambon P, Ko C 2008 Pituitary gonadotroph estrogen receptor-alpha is necessary for fertility in females. Endocrinology 149:20-27
114. Belchetz PE, Plant TM, Nakai Y, Keogh EJ, Knobil E 1978 Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone. Science 202:631-633
115. de Kretser DM, Buzzard JJ, Okuma Y, O'Connor AE, Hayashi T, Lin SY, Morrison JR, Loveland KL, Hedger MP 2004 The role of activin, follistatin and inhibin in testicular physiology. Mol Cell Endocrinol 225:57-64
116. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A 90:11162-11166
117. Nakao N, Ono H, Yamamura T, Anraku T, Takagi T, Higashi K, Yasuo S, Katou Y, Kageyama S, Uno Y, Kasukawa T, Iigo M, Sharp PJ, Iwasawa A, Suzuki Y, Sugano S, Niimi T, Mizutani M, Namikawa T, Ebihara S, Ueda HR, Yoshimura T 2008 Thyrotrophin in the pars tuberalis triggers photoperiodic response. Nature 452:317-322
118. Ono H, Hoshino Y, Yasuo S, Watanabe M, Nakane Y, Murai A, Ebihara S, Korf HW, Yoshimura T 2008 Involvement of thyrotropin in photoperiodic signal transduction in mice. Proc Natl Acad Sci U S A 105:18238-18242
119. Rennstam K, McMichael N, Berglund P, Honeth G, Hegardt C, Ryden L, Luts L, Bendahl PO, Hedenfalk I 2009 Numb protein expression correlates with a basal-like phenotype and cancer stem cell markers in primary breast cancer. Breast Cancer Res Treat 122(2):315-24
120. Singh SP, Wolfe A, Ng Y, DiVall SA, Buggs C, Levine JE, Wondisford FE, Radovick S 2009 Impaired estrogen feedback and infertility in female mice with pituitary-specific deletion of estrogen receptor alpha (ESR1). Biol Reprod 81:488-496
121. Yin P, Kawashima K, Arita J 2002 Direct actions of estradiol on the anterior pituitary gland are required for hypothalamus-dependent lactotrope proliferation and

secretory surges of luteinizing hormone but not of prolactin in female rats. Neuroendocrinology 75:392-401
122. Mitchner NA, Garlick C, Ben-Jonathan N 1998 Cellular distribution and gene regulation of estrogen receptors alpha and beta in the rat pituitary gland. Endocrinology 139:3976-3983
123. Sanchez-Criado JE, de Las Mulas JM, Bellido C, Aguilar R, Garrido-Gracia JC 2005 Gonadotrope oestrogen receptor-alpha and -beta and progesterone receptor immunoreactivity after ovariectomy and exposure to oestradiol benzoate, tamoxifen or raloxifene in the rat: correlation with LH secretion. J Endocrinol 184:59-68
124. Sanchez-Criado JE, Martin De Las Mulas J, Bellido C, Tena-Sempere M, Aguilar R, Blanco A 2004 Biological role of pituitary estrogen receptors ERalpha and ERbeta on progesterone receptor expression and action and on gonadotropin and prolactin secretion in the rat. Neuroendocrinology 79:247-258
125. Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development 127:4277-4291
126. Hewitt SC, Korach KS 2003 Oestrogen receptor knockout mice: roles for oestrogen receptors alpha and beta in reproductive tissues. Reproduction 125:143-149
127. Watson CS, Campbell CH, Gametchu B 2002 The dynamic and elusive membrane estrogen receptor-alpha. Steroids 67:429-437
128. Watson CS, Pappas TC, Gametchu B 1995 The other estrogen receptor in the plasma membrane: implications for the actions of environmental estrogens. Environ Health Perspect 103 Suppl 7:41-50
129. Burbach KM, Poland A, Bradfield CA 1992 Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Proc Natl Acad Sci U S A 89:8185-8189
130. Ema M, Sogawa K, Watanabe N, Chujoh Y, Matsushita N, Gotoh O, Funae Y, Fujii-Kuriyama Y 1992 cDNA cloning and structure of mouse putative Ah receptor. Biochem Biophys Res Commun 184:246-253
131. Baglole CJ, Maggirwar SB, Gasiewicz TA, Thatcher TH, Phipps RP, Sime PJ 2008 The aryl hydrocarbon receptor attenuates tobacco smoke-induced cyclooxygenase-2 and prostaglandin production in lung fibroblasts through regulation of the NF-kappaB family member RelB. J Biol Chem 283:28944-28957
132. Schecter A, Birnbaum L, Ryan JJ, Constable JD 2006 Dioxins: an overview. Environ Res 101:419-428
133. Carver LA, Bradfield CA 1997 Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog in vivo. J Biol Chem 272:11452-11456
134. Ma Q, Whitlock JP,Jr 1997 A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Biol Chem 272:8878-8884
135. Bacsi SG, Hankinson O 1996 Functional characterization of DNA-binding domains of the subunits of the heterodimeric aryl hydrocarbon receptor complex imputing novel and canonical basic helix-loop-helix protein-DNA interactions. J Biol Chem 271:8843-8850

136. Swanson HI, Chan WK, Bradfield CA 1995 DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. J Biol Chem 270:26292-26302
137. Sharara FI, Seifer DB, Flaws JA 1998 Environmental toxicants and female reproduction. Fertil Steril 70:613-622
138. Miller KP, Borgeest C, Greenfeld C, Tomic D, Flaws JA 2004 In utero effects of chemicals on reproductive tissues in females. Toxicol Appl Pharmacol 198:111-131
139. Matikainen T, Perez GI, Jurisicova A, Pru JK, Schlezinger JJ, Ryu HY, Laine J, Sakai T, Korsmeyer SJ, Casper RF, Sherr DH, Tilly JL 2001 Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. Nat Genet 28:355-360
140. Mattison DR, Singh H, Takizawa K, Thomford PJ 1989 Ovarian toxicity of benzo(a)pyrene and metabolites in mice. Reprod Toxicol 3:115-125
141. Gray LE,Jr, Ostby JS 1995 In utero 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters reproductive morphology and function in female rat offspring. Toxicol Appl Pharmacol 133:285-294
142. Flaws JA, Sommer RJ, Silbergeld EK, Peterson RE, Hirshfield AN 1997 In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces genital dysmorphogenesis in the female rat. Toxicol Appl Pharmacol 147:351-362
143. Abbott BD, Schmid JE, Pitt JA, Buckalew AR, Wood CR, Held GA, Diliberto JJ 1999 Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse. Toxicol Appl Pharmacol 155:62-70
144. Baba T, Mimura J, Nakamura N, Harada N, Yamamoto M, Morohashi K, Fujii-Kuriyama Y 2005 Intrinsic function of the aryl hydrocarbon (dioxin) receptor as a key factor in female reproduction. Mol Cell Biol 25:10040-10051
145. Benedict JC, Lin TM, Loeffler IK, Peterson RE, Flaws JA 2000 Physiological role of the aryl hydrocarbon receptor in mouse ovary development. Toxicol Sci 56:382-388
146. Benedict JC, Miller KP, Lin TM, Greenfeld C, Babus JK, Peterson RE, Flaws JA 2003 Aryl hydrocarbon receptor regulates growth, but not atresia, of mouse preantral and antral follicles. Biol Reprod 68:1511-1517
147. Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, Tohyama C, Krust A, Mimura J, Chambon P, Yanagisawa J, Fujii-Kuriyama Y, Kato S 2003 Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature 423:545-550
148. Barnes-Ellerbe S, Knudsen KE, Puga A 2004 2,3,7,8-Tetrachlorodibenzo-p-dioxin blocks androgen-dependent cell proliferation of LNCaP cells through modulation of pRB phosphorylation. Mol Pharmacol 66:502-511
149. Gregoraszczuk EL, Wojtowicz AK, Zabielny E, Grochowalski A 2000 Dose-and-time dependent effect of 2,3,7,8-tetrachlorodibenzo-P-dioxin (TCDD) on progesterone secretion by porcine luteal cells cultured in vitro. J Physiol Pharmacol 51:127-135
150. Heliovaara E, Raitila A, Launonen V, Paetau A, Arola J, Lehtonen H, Sane T, Weil RJ, Vierimaa O, Salmela P, Tuppurainen K, Makinen M, Aaltonen LA, Karhu A 2009 The expression of AIP-related molecules in elucidation of cellular pathways in pituitary adenomas. Am J Pathol 175:2501-2507

151. Buchbinder S, Bierhaus A, Zorn M, Nawroth PP, Humpert P, Schilling T 2008 Aryl hydrocarbon receptor interacting protein gene (AIP) mutations are rare in patients with hormone secreting or non-secreting pituitary adenomas. Exp Clin Endocrinol Diabetes 116:625-628
152. Yu R, Bonert V, Saporta I, Raffel LJ, Melmed S 2006 Aryl hydrocarbon receptor interacting protein variants in sporadic pituitary adenomas. J Clin Endocrinol Metab 91:5126-5129
153. Vanden Heuvel JP, Clark GC, Thompson CL, McCoy Z, Miller CR, Lucier GW, Bell DA 1993 CYP1A1 mRNA levels as a human exposure biomarker: use of quantitative polymerase chain reaction to measure CYP1A1 expression in human peripheral blood lymphocytes. Carcinogenesis 14:2003-2006
154. Stephen FD, Drahushuk AT, Olson JR 1997 Cytochrome P450 1A1 induction in rat lymphoid tissues following in vivo and in vitro exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin requires protein kinase C. Toxicology 124:39-51
155. Spink BC, Fasco MJ, Gierthy JF, Spink DC 1998 12-O-tetradecanoylphorbol-13-acetate upregulates the Ah receptor and differentially alters CYP1B1 and CYP1A1 expression in MCF-7 breast cancer cells. J Cell Biochem 70:289-296
156. Liu L, Merriam GR, Sherins RJ 1987 Chronic sex steroid exposure increases mean plasma growth hormone concentration and pulse amplitude in men with isolated hypogonadotropic hypogonadism. J Clin Endocrinol Metab 64:651-656
157. Shull JD, Walent JH, Gorski J 1987 Estradiol stimulates prolactin gene transcription in primary cultures of rat anterior pituitary cells. J Steroid Biochem 26:451-456
158. Abbott BD, Birnbaum LS 1990 TCDD-induced altered expression of growth factors may have a role in producing cleft palate and enhancing the incidence of clefts after coadministration of retinoic acid and TCDD. Toxicol Appl Pharmacol 106:418-432
159. Gonzalez FJ, Fernandez-Salguero P 1998 The aryl hydrocarbon receptor: studies using the AHR-null mice. Drug Metab Dispos 26:1194-1198
160. Barnett KR, Tomic D, Gupta RK, Babus JK, Roby KF, Terranova PF, Flaws JA 2007 The aryl hydrocarbon receptor is required for normal gonadotropin responsiveness in the mouse ovary. Toxicol Appl Pharmacol 223:66-72
161. Shimba S, Komiyama K, Moro I, Tezuka M 2002 Overexpression of the aryl hydrocarbon receptor (AhR) accelerates the cell proliferation of A549 cells. J Biochem 132:795-802
162. Mulero-Navarro S, Pozo-Guisado E, Perez-Mancera PA, Alvarez-Barrientos A, Catalina-Fernandez I, Hernandez-Nieto E, Saenz-Santamaria J, Martinez N, Rojas JM, Sanchez-Garcia I, Fernandez-Salguero PM 2005 Immortalized mouse mammary fibroblasts lacking dioxin receptor have impaired tumorigenicity in a subcutaneous mouse xenograft model. J Biol Chem 280:28731-28741
163. Nannelli A, Rossignolo F, Tolando R, Rossato P, Longo V, Gervasi PG 2009 Effect of beta-naphthoflavone on AhR-regulated genes (CYP1A1, 1A2, 1B1, 2S1, Nrf2, and GST) and antioxidant enzymes in various brain regions of pig. Toxicology 265:69-79
164. Gauger KJ, Giera S, Sharlin DS, Bansal R, Iannacone E, Zoeller RT 2007 Polychlorinated biphenyls 105 and 118 form thyroid hormone receptor agonists after cytochrome P4501A1 activation in rat pituitary GH3 cells. Environ Health Perspect 115:1623-1630

165. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC 2009 Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev 30:293-342
166. Takeda T, Matsumoto Y, Koga T, Mutoh J, Nishimura Y, Shimazoe T, Ishii Y, Ishida T, Yamada H 2009 Maternal exposure to dioxin disrupts gonadotropin production in fetal rats and imprints defects in sexual behavior. J Pharmacol Exp Ther 329:1091-1099
167. Mutoh J, Taketoh J, Okamura K, Kagawa T, Ishida T, Ishii Y, Yamada H 2006 Fetal pituitary gonadotropin as an initial target of dioxin in its impairment of cholesterol transportation and steroidogenesis in rats. Endocrinology 147:927-936
168. Zaher H, Fernandez-Salguero PM, Letterio J, Sheikh MS, Fornace AJ, Jr, Roberts AB, Gonzalez FJ 1998 The involvement of aryl hydrocarbon receptor in the activation of transforming growth factor-beta and apoptosis. Mol Pharmacol 54:313-321
169. Sarkar DK, Kim KH, Minami S 1992 Transforming growth factor-beta 1 messenger RNA and protein expression in the pituitary gland: its action on prolactin secretion and lactotropic growth. Mol Endocrinol 6:1825-1833
170. Ben-Jonathan N, Hnasko R 2001 Dopamine as a prolactin (PRL) inhibitor. Endocr Rev 22:724-763

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Dissertation Adviser: Dr. Lori Raetzman

Bates College, Lewiston, ME. Neuroscience (B.S.) Honors**,** May 2002

Teaching Experience

- **Teaching Assistant, Medical Neuroscience.** Led first year medical students in small group discussions during neuroscience interactive learning experience (NILE). Spring 2008, 2009.
- **Teaching Assistant, Histology.** Identified histological features and taught basic microscope skills to first year medical students during course laboratory session. Fall 2010.

Mentoring Experience

- Mentored and managed four undergraduate research assistants, 2005-2009.
- Advised incoming graduate students in the Raetzman Lab, 2005-2010.

Research Experience

Doctoral student, Laboratory of Lori Raetzman, 2005-2010

- Identified novel expression of the protein Numb in the mouse pituitary by immunohistochemistry, *in situ* hybridization, and reverse transcriptase PCR.
- Determined function of Numb in the mouse pituitary by using conditional knockout mouse model.
- Investigated endocrine disrupting actions of aryl hydrocarbon receptor agonists on pituitary cell cultures.

Lab Manager / Research Technician Children's Hospital Boston and Dana-Farber Cancer Institute, Boston, MA 2002-2005

- Performed lab manager duties and helped establish Cantor Lab at Children's Hospital Boston
- Performed multi-protein complex isolation and identification using cell culture, hematopoietic stem cell transfection and viral transduction, and HPLC.

Undergraduate Research Experience

- Year-long senior thesis research entitled *A Role of Serotonin in a PCP Model of Schizophrenia.* Bates College, Lewiston, ME.
- Selected for Career Discovery Internship Program in diabetes research at Yale University School of Medicine, February 2000. New Haven, CT.

Publications:

Moran TB, Goldberg L, Serviss S, Raetzman LT. Numb deletion in POMC expressing cells impairs pituitary intermediate lobe cell adhesion, progenitor cell localization, and neuro-intermediate lobe boundary formation. *Accepted for publication in Molecular Endocrinology, 2011.*

Yu M, Riva L, Xie H, Schindler Y, **Moran TB**, Cheng Y, Yu D, Hardison R, Weiss MJ, Orkin SH, Bernstein BE, Fraenkel E, Cantor AB. Insights into GATA-1-mediated gene activation versus repression via genome-wide chromatin occupancy analysis. *Mol Cell***,** 2009 Nov 25;36(4):682-95.

Amigo JD, Ackermann GE, Cope JJ, Yu M, Cooney JD, Ma D, Langer NB, Shafizadeh E, Shaw GC, Horsely W, Trede NS, Davidson AJ, Barut BA, Zhou Y, Wojiski SA, Traver D, **Moran TB**, Kourkoulis G, Hsu K, Kanki JP, Shah DI, Lin HF, Handin RI, Cantor AB, Paw BH. The role and regulation of friend of GATA-1 (FOG-1) during blood development in the zebrafish. *Blood*. 2009 Nov 19;114(21):4654-63.

Huang H, Yu M, Akie TE, **Moran TB**, Woo AJ, Tu N, Waldon Z, Lin YY, Steen H, Cantor AB. Differentiation-dependent interactions between RUNX-1 and FLI-1 during megakaryocyte development. *Mol Cell Biol*. 2009 Aug;29(15):4103-15.

Cantor AB, Iwasaki H, Arinobu Y, **Moran TB**, Shigematsu H, Sullivan MR, Akashi K, Orkin SH. Antagonism of FOG-1 and GATA factors in fate choice for the mast cell lineage. *J Exp Med*. 2008 Mar 17;205(3):611-24

Woo AJ, **Moran TB**, Schindler YL, Choe SK, Langer NB, Sullivan MR, Fujiwara Y, Paw BH, Cantor AB. Identification of ZBP-89 as a novel GATA-1-associated transcription factor involved in megakaryocytic and erythroid development. *Mol Cell Biol*. 2008 Apr;28(8):2675-89.

Pal S, Cantor AB, Johnson KD, **Moran TB**, Boyer ME, Orkin SH, Bresnick EH. Coregulator-dependent facilitation of chromatin occupancy by GATA-1. *Proc Natl Acad Sci U S A*. 2004 Jan 27;101(4):980-5

Abstracts:

Moran, T.B., Goldberg, L.B., Serviss, S., and Raetzman, L.T. (2010) Numb, an endocytic adaptor protein is critical to maintain cell adhesion in the intermediate lobe of the mouse pituitary. *49th Annual Midwest Developmental Biology Meeting*, Cincinnati, Ohio.

Goldberg, L., **Moran, T.B**., Brannick, K., and Raetzman, L.T. (2010) The Notch Inhibitor Numb is Present in Gonadotropes and May be Necessary for LH Expression and Function. *43rd Society for the Study of Reproduction Meeting*. Milwaukee, WI.

Moran, T.B., Raetzman, L.T. (2009) Aryl Hydrocarbon Agonists Modulate Proliferation and Impair Hormone Synthesis in the Pituitary. *The Endocrine Society's 91st Annual Meeting*, Washington, DC.

Woo, A., **Moran,** T.B., Choe, S., Schindler, Y., Sullivan, S., Fujiwara, Y., Paw, B.H., Cantor, A.B. (2005) Identification of zfp148 (ZBP-89) as a novel GATA-1 associated transcription factor required for megakaryopoiesis and definitive erythropoiesis. *47th Annual Meeting of the American Society of Hematology*, Atlanta, Georgia.

Moran, T.B. Choe, S., Paw, B.H., Cantor, A.B. (2004) GATA-1 multiprotein purification: identification of zfp148 as a novel GATA-1 associated protein required for megakaryopoiesis. Poster presented at *Fourteenth Conference on Hemoglobin Switching*, Orcas Island, Washington.

Kelsey, J.E., **Moran, T**., and Winterton, J. (2002) The role of serotonin in PCP-induced behaviors in the hole board apparatus. *Society for Neuroscience Abstracts*, 28, Program No. 494.11.

University Service

College of Medicine

- **Medical Scholars Program Advisory Committee (MSPAC)**
  - Admissions Committee, student representative 2009-2010
  - Class Representative, 2009-2010
- **College of Medicine Research Day Planning Committee**
  - Participated during 2006-2010
- **Medical Scholars Program Retreat Planning Committee**
  - Participated during 2006-2010

Community Outreach

- **Brain Awareness Day volunteer**; *The Doctor is In*. Described cranial nerves at open public event and performed basic cranial nerve exams. April 2006, 2007-2010. Lincoln Square Mall, Urbana, IL

- Managed a table at 15th Annual Kid's Building Fair May 2007. Orpheum Children's Science Museum, Champaign, IL

Fellowships:

- Hazel I. Craig Fellowship, 2009

References available upon request